INTERACTION OF TETRACYCLINE WITH PROTEIN SYNTHESIZING SYSTEM OF *STREPTOMYCES AUREOFACIENS*

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The interaction between protein synthesis and production of tetracycline in *Streptomyces aureofaciens* was studied. It has been shown that the amount of tetracycline bound to ribosomes rapidly rises, as compared with the production of the drug. The accumulation of tetracycline in the cultivation medium results in the formation of tetracycline-ribosome aggregates. The highest level of binding was equivalent to 320 molecules of tetracycline per ribosome. The results obtained with the S 30 fraction, containing endogenous mRNA suggest that protein-synthesizing system of tetracycline-producing microorganisms is more resistant to the antibiotic effect than similar systems isolated from drug-sensitive bacteria. Tetracycline itself can have a regulatory function in the metabolism of producing cells. Accumulation of tetracycline during growth may lead to limitation of protein synthesis and enzyme systems involved in the machinery of secondary metabolite biosynthesis.

The relationship between antibiotic synthesis and other metabolic processes of the producing cell has been the subject of numerous discussions. One approach to this problem is to correlate the formation of the antibiotics with the protein synthesizing machinery of the producing microorganism. As an experimental model we used *Streptomyces aureofaciens* which synthesizes a broad spectrum of metabolites, including antibiotics such as tetracycline and chlortetracycline.

This group of drugs interacts with a wide variety of reactions, involving respiration[1,2] synthesis[3~6] and activities of enzymes[7~13].

During the past few years there have been a number of reports about the inhibitory effect of tetracyclines on protein synthesis; GURGO *et al.*[14] and CUNDLIFFE[15] have reported that the addition of tetracycline to *Escherichia coli* cells caused an inhibition of polysome formation and an accumulation of free 30 S and 50 S ribosomal subunits. Data from several laboratories have indicated that tetracyclines prevent the binding of aminoacyl-tRNA to the aminoacyl acceptor site of ribosomes[16~19]. However, higher concentrations of tetracycline inhibit also the release of deacylated tRNA from ribosomes[20,21].

The purpose of the experiments described in this communication was to study the interaction of tetracycline with protein synthesizing machinery of *S. aureofaciens*, which produces under laboratory condition about 200 times more of the drug than the

amount required for total inhibition of growth of sensitive bacterial cells.

Material and Methods

Streptomyces aureofaciens, tetracycline-producing strain (84/25) was obtained from the Research Institute of Antibiotics and Biotransformations (Prague). The cultures were grown at 28 °C in 500-ml flasks containing 60 ml media[22]. The cells were harvested at different stages of growth and washed by suspension in a cold buffer containing 10 mM Tris-HCl pH 7.5, 10 mM magnesium acetate, 60 mM KCl and 6 mM 2-mercaptoethanol (TCMK).

In vivo ^{14}C-leucine incorporation into proteins

Radioactive U^{14}C-leucine, specific activity 83 mC/mMol was added to the cultures of *S. aureofaciens* in an amount of 20 μC/60 ml medium. In experiments following the effect of tetracycline on ^{14}C-leucine incorporation, 50~1,000 μg/ml tetracycline was supplied at 4 hours of cultivation. One ml samples were withdrawn at different intervals of growth and poured into 2 ml of 10 % TCA. Chilled samples were filtered and washed on membrane filters. Radioactivity was measured on a Biospan Nuclear Chicago Counter.

In vivo ^{14}C-thymine incorporation into DNA

Radioactive ^{14}C-thymine (specific activity 50 mC/mMol), was added at the beginning of the cultivation of *S.aureofaciens* in an amount of 20 μC/60 ml medium. One ml aliquots were poured into 2 ml of ice cold 5% TCA, filtered and washed on membrane filters. Radioactivity was measured as in the experiments with radioactive amino acid.

Preparation of ribosomes and ribosomal subunits

Isolation of ribosomes from alumina-ground cells is described elsewhere[23]. The ribosomal pellet was washed twice with TCMK buffer pH 7.6, and the ribosomes purified by centrifugation through 30% sucrose in the same buffer. The purification procedure was repeated twice. The ribosomes were dissociated into subunits by dialysis against TCMK buffer containing 0.1 mM magnesium acetate. The ribosomal subunits were layered over 28 ml 10~30 % sucrose gradient and centrifuged in an SW 25.1 rotor for 15$^1/_2$ hours at 19,600 r.p.m. on a Spinco model L2-65 B centrifuge. Separated subunits from 3 tubes were combined and centrifuged for 5 hours at 48,000 r.p.m. in a 50.1 rotor. The separation procedure was repeated twice.

Gel filtration of ribosomal aggregates

Ribosomes, 3.75 mg, were incubated in a mixture containing 10 mM Tris-HCl buffer pH 7.5, 10 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM 2-mercaptoethanol and 1 mM ^{3}H-tetracycline (1 μC). After 90-minute incubation at 30°C, the mixture was centrifuged at 105,000 $\times g$ for 30 minutes, the supernatant discarded and the ribosomal aggregates washed with 10 ml of the above buffer to remove free tetracycline. The resulting sediment was suspended in 1 ml of buffer and passed through a 2 $\times$ 50 cm Sephadex G 50 column.

Aminoacyl synthetase preparation

Aminoacyl synthetase activity was estimated in a partially purified 105,000 $\times g$ supernatant fraction[24]. The supernatant fraction was stirred and 0.2 ml of 5 % streptomycin sulfate solution slowly added. After 15 minutes of stirring, the precipitated material was removed by centrifugation at 10,000 $\times g$. The sediment was removed and the supernatant adjusted to pH 5.2 with 0.1 N HCl. The mixture was centrifuged at 10,000 $\times g$ for 10 minutes. The supernatant was discarded and the sediment suspended in 0.1 M Tris-HCl buffer (pH 7.6) containing 6 mM dithiothreitol. After 8-hour dialysis against the same buffer the solution was centrifuged at 15,000 $\times g$ for 10 minutes. The supernatant solution was mixed 1 : 1 with glycerol and stored until use at -20°C. This solution was used as a source of aminoacyl synthetase activity.

Cell-free amino acid incorporating system

The activity of the S 30 fraction and the effect of tetracycline were assayed using an *in vitro* ^{14}C-valine-incorporating system of MATTHAEI and NIRENBERG[24]. The specific activity

of L-^{14}C-valine (New England Nuclear Corp.) was 50 mC/mMol. The reaction mixtures were incubated at 37°C for 25 minutes and then treated with 3 ml of 10 % TCA. The samples were heated in a water bath for 20 minutes at 90°C, then chilled in ice and filtered under suction through a membrane filter. The radioactivity of the dried filters was measured.

Sedimentation velocity analysis

This was done in a Spinco model E analytical ultracentrifuge. The sedimentation was studied at 37,020 r.p.m. and 20°C. Bar angle 60°.

Preparation of ribosomal proteins

Ribosomal proteins were prepared from purified ribosomes by the LiCl-urea method of TRAUB and NOMURA[25].

Electrophoresis

Polyacrylamide disc electrophoresis of ribosomal proteins was carried out by the method of GESTELAND and STAEHELIN[26]. The tube size was 0.6×7.0 cm. The samples were added on top of the upper gel, along with 1 μl of 0.1 % pyronine Y (FISCHER, P 388). Electrophoresis was done in the cold, for about 3 hours at 5 mA/tube. The gels were stained with amidoblack 10 B (Merck) used as a 0.5 % solution in 7.5 % acetic acid.

Results and Discussion

The first question studied was whether tetracycline can pass through the cell wall of *S. aureofaciens* and interfere with two essential steps of cell metabolism, *i.e.* protein synthesis and synthesis of DNA. Fig. 1 shows the course of synthesis of DNA (measured as incorporation of ^{14}C-thymine into DNA), incorporation of ^{14}C-leucine into proteins and production of tetracycline. Incorporation of ^{14}C-thymine as well as ^{14}C-leucine increased up to 12 hours of cultivation, while the concentration of tetracycline in the fermentation medium remained relatively low (50 μg/ml). The later increase in tetracycline production was accompanied by a decrease of radioactivity of both DNA and

Fig. 1. The course of ^{14}C-leucine incorporation into the proteins, the ^{14}C-thymine incorporation into DNA and production of tetracycline.
For experimental details, see Material and Methods.

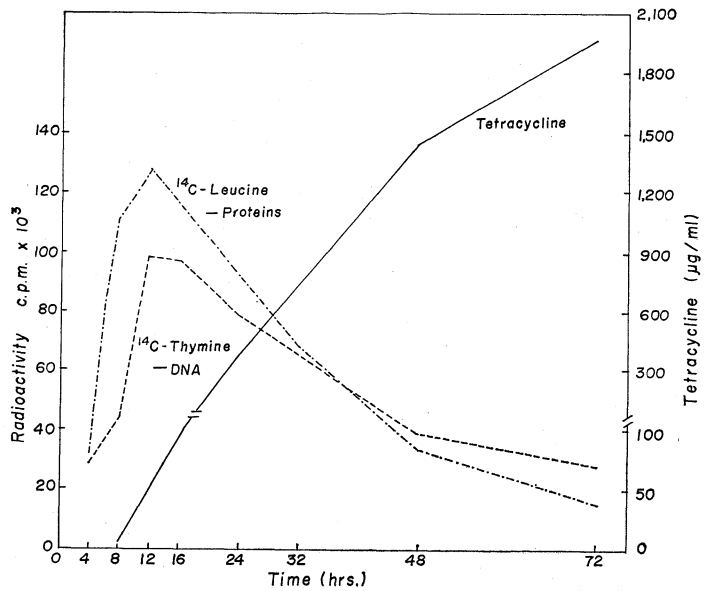


Fig. 2. The effect of addition of tetracycline at different intervals of growth of *S. aureofaciens* on ^{14}C-leucine and ^{14}C-thymine incorporation and on production of the antibiotic.

A : the course of ^{14}C-leucine incorporation into proteins after addition of 500 μg/ml tetracycline at 16 hours of cultivation
B : the course of tetracycline production before and after addition of 500 μg/ml tetracycline at 16 hours of cultivation
C : the course of ^{14}C-leucine incorporation after addition of 500 μg/ml tetracycline to 4-hour culture
D : the course of ^{14}C-thymine incorporation under the same experimental condition
E : the level of tetracycline after addition of 500 μg/ml tetracycline to 4-hour culture.

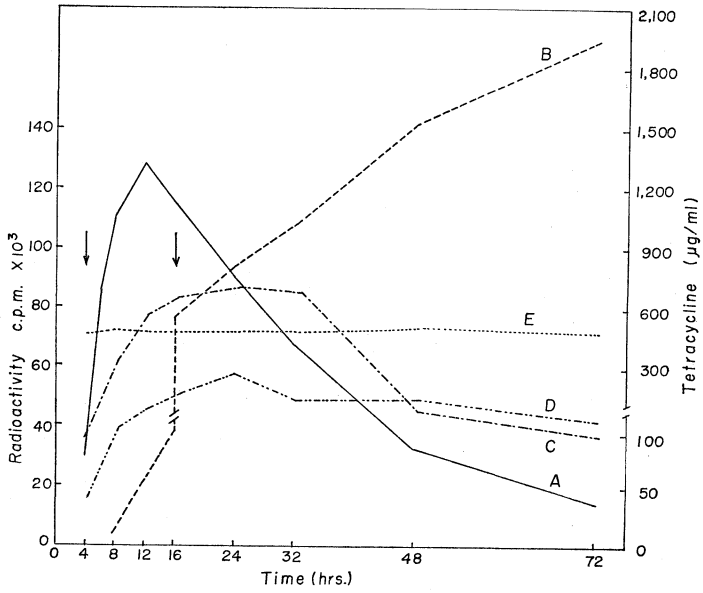
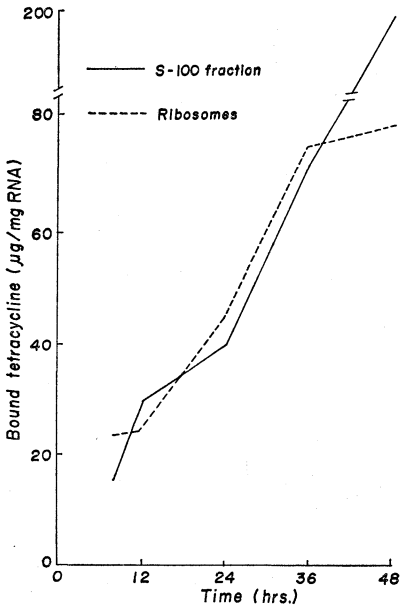


Fig. 3. Distribution of tetracycline among the subcellular fraction.
Ribosomes and S-100 fraction were prepared as described in the Material and Methods section.



proteins. At 72 hours of cultivation, the accumulation of tetracycline was about 2,000 μg/ml.

The effect of tetracycline on cultures at different stages of growth was then studied. The addition of 500 μg/ml tetracycline to a 4-hour culture (Fig. 2) resulted in a 37 % inhibition of ^{14}C-leucine incorporation into proteins, a 45 % inhibition of ^{14}C-thymine incorporation to DNA and a shift of the maximum incorporation to about 12 hours. The production of tetracycline was totally inhibited.

When the drug was added to a 16-hour culture under the same experimental condition, the synthesis of tetracycline was not affected. These results indicate that tetracycline interferes with the synthesis of specific enzymes which are needed for the biosynthesis of the antibiotic rather than with their activities.

Intracellular localization of tetracycline, sedimentation and electrophoretical pattern of the ribosomes

Fig. 4. Sedimentation profile of crude ribosomal preparations isolated from 8, 12, 24, 48 and 72 hours of cultivation of the tetracycline producing strain.
Each sample contains 2,5 mg proteins. Centrifugations were performed in a Spinco Model E analytical ultracentrifuge at 37,020 r.p.m. at 20℃, using a 60° bar angle.

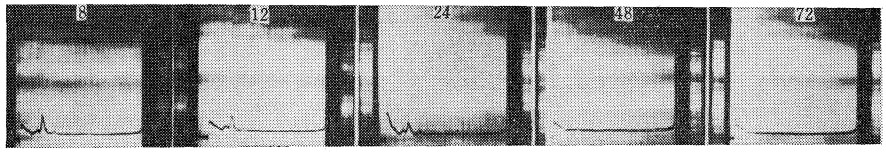
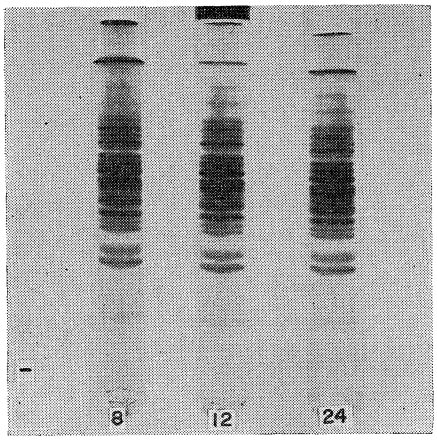


Fig. 5. Electrophoretic pattern of ribosomal proteins isolated from ribosomes of 8, 12 and 24-hour cultures of *S. aureofaciens*.
Electrophoresis was carried out at pH 4,5. in the cold at 5 mA/column.



Further experiments were performed in order to ascertain whether intracellular accumulation of tetracycline during the growth of a tetracycline-producing strain of *S. aureofaciens* is accompanied by changes in the sedimentation properties of ribosomes. The results presented in Fig. 3 indicate that the amount of tetracycline bound to subcellular fractions rose rapidly at about 24 hours of cultivation, comparable with production of the antibiotic (Fig. 1). The sedimentation profile of the ribosomes remained consistent up to 24 hours of growth; *i.e.* up to the time when the concentration of tetracycline in the cultivation medium did not exceed 500 μg/ml (Fig. 4). Moreover, the electrophoretic patterns of ribosomal proteins isolated from ribosomes of 8, 12 and 24-hour cultures of the producing strain also remained unaltered (Fig. 5). The preparation isolated from 48 and 72-hour cultures contain ribosomal aggregates which sediment rapidly. Electronoptic evidence for the formation of such ribosomal aggregates in the presence of higher concentrations of chlortetracycline (1 mM) has been reported in a previous paper[27].

Binding of tetracycline to ribosomes and ribosomal subunits

It has been reported that tetracycline binds to 70 S ribosomes[28~30] and ribosomal usbunits. The amount of tetracycline bound to 30S subunits is considerably greater than to 50S subunits[31]. This was confirmed in our experiments with the ribosomes of *S. aureofaciens*. Five hundreds μg/ml of ^{3}H-tetracycline (20 μC) were added to a 10-hour culture. After 2 hours of cultivation at 28°C, the culture was chilled, washed twice with TCMK buffer (pH 7.5). The ribosomes isolated were dialyzed against TCMK buffer containing either 10 mM or 0.1 mM magnesium acetate. Ribosomes and ribosomal subunits were fractionated into sucrose density gradients. The results presented as Fig. 6 show that radioactivity of ^{3}H-tetracycline was associated with 70S and 100S dimers. In experiments using 0.1 mM Mg^{2+} the main radioactivity from tetracycline was associated with the 30S subunits. A small but significant radioactivity is associated with the 50S particles. Considerable radioactivity was found with the ribosomal aggregates sedimenting at the bottom of the centrifugation tubes. These agregates are stable when treated with trypsin or pancreatic ribonuclease. The

Fig. 6. Binding of ^{3}H-tetracycline to ribosomes and ribosomal subunits of the tetracycline-producing strain of *S. aureofaciens*.

Cells of 10-hour cultures were treated for 2 hours with ^{3}H-tetracycline 500 μg/ml (20 μC). 20 A_{260} units of ribosomal subunits (B) and 19 A_{260} units of ribosomes (A) were layered on 10~30% sucrose gradients and centrifuged for 15½ hours at 19,600 r.p.m. One ml fractions were collected. The sediment remaining at the bottom of the centrifugation tubes was suspended in 1 ml of TCMK buffer pH 7.5.

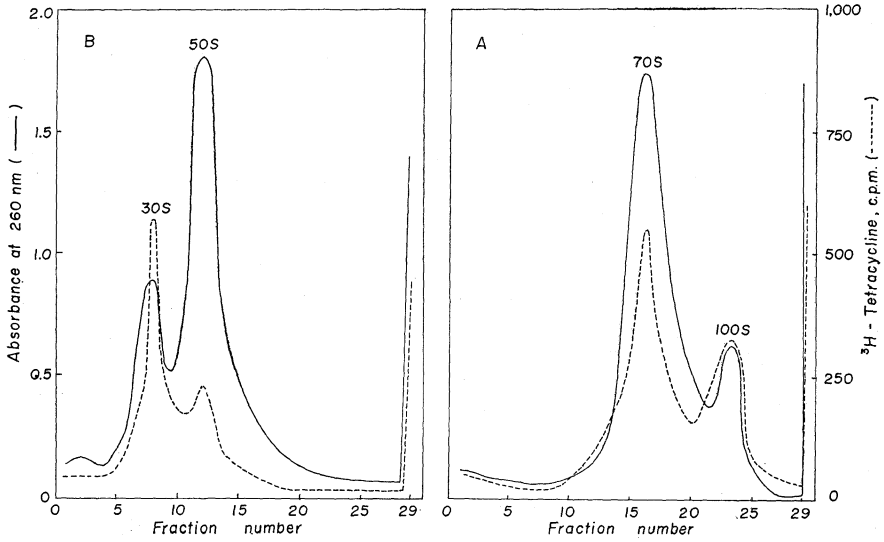
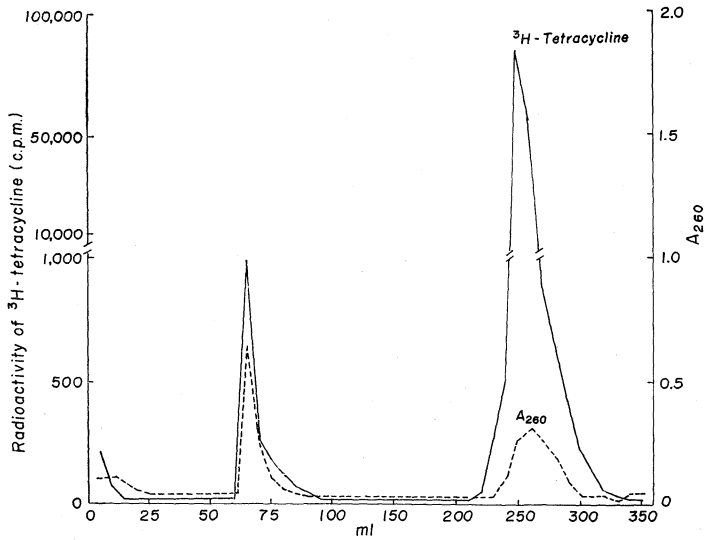


Fig. 7. Sephadex G 50 gel filtration of tetracycline-ribosome complex.

washed complex was applied on a 2×50 cm Sephadex G 50 column and eluted with 10 mM Tris-HCl buffer pH 7.5, containing 10 mM $MgCl_2$, 30 mM NH_4Cl and 6 mM 2-mercaptoethanol.



maximum level of binding observed was equivalent to 320 molecules of tetracycline per ribosome. This value was calculated from the absorbance and radioactivity of the 70S peak fraction. The binding of tetracycline to the ribosomes of *S. aureofaciens* was largely reversible, like that reported for the ribosomes of *Bacillus megatherium*[31].

Using ^{3}H-tetracycline of high specific activity, it wsa possible to detect a level of irreversible binding. Fig. 7 shows an elution profile of the tetracycline-ribosome

complex from a Sephadex G50 column. About 1% of the total radioactivity was connected with the ribosomes. It was concluded that about one molecule tetracycline was tightly bound to each ribosome.

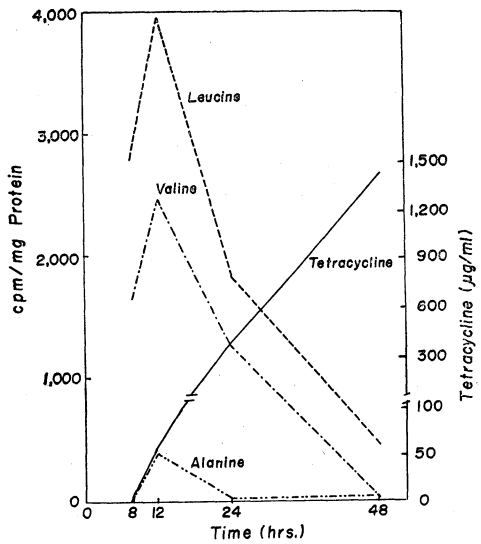
Activation of amino acids and *in vitro* protein synthesis by the tetracycline-producing strain of *S. aureofaciens*

Aminoacyl-tRNA synthetases fulfil two important roles in protein synthesis; *i.e.* activation of amino acids and translation of specific base sequences in the mRNA into amino acid specificity.

In order to obtain more detailed information about relationships between the initial steps of protein synthesis and the production of tetracycline, the charging of *E. coli* tRNAs with radioactive leucine, valine and alanine was estimated. These reactions were catalyzed by aminoacyl synthetases, isolated at different stages of growth of *S. aureofaciens*. The concentration of protein in these partially purified enzyme mixtures was adjusted to 1 mg/ml. The maximum aminoacyl synthetase activity for all substrates studied was observed with preparations isolated from 12-hour cultures (Fig. 8). There exists a direct if not a proportional relationship between the depression of aminoacyl synthetase activity and the production of the antibiotic. Similar conclusions were reported by HOŠŤÁLEK *et al.*[32] who studied interaction of individual enzymes of the tricarboxylic acid cycle and production of tetracycline by *S. aureofaciens*.

Fig. 8. Activity of aminoacyl-tRNA synthetases at different intervals of growth and the production of tetracycline.

Partially purified preparations of aminoacyl synthetases (see Material and Methods) were incubated in a reaction mixture containing 0.1 M Tris-HCl buffer pH 7.5, 0.01 M KCl, 0.02 M magnesium acetate, 0.005 M ATP, 0.005 M CTP, 8 A_{260} units of *E. coli* tRNA, 0.003 M mixture of unlabelled amino acid, plus 0.012 μ moles of each of the ^{14}C-amino acid tested. Incubation: 15 minutes at 37°C. The reactions were stopped by adding 2 ml of cold 10% TCA. The samples were filtered through a membrane filter, washed three times with 5 ml of ice-cold 5% TCA and radioactivity measured.



Next, we studied the effect of tetracycline on an *in vitro* protein-synthesizing system. Table 1 shows the composition of the system prepared from 10-hour cultures of *S. aureofaciens*. The incorporation of ^{14}C-valine into proteins was proportional to the amount of S30 fraction added. The

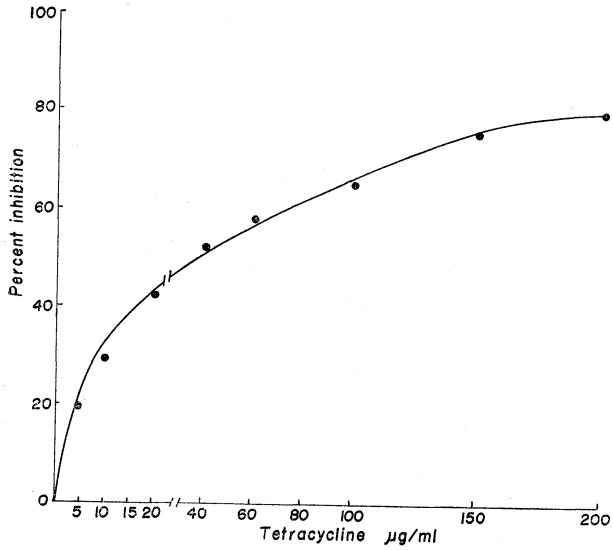
Table 1. Characteristics of *in vitro* portein synthesis in *Streptomyces aureofaciens* directed by endogenous mRNA

Incubation components	Radioactivity c. p. m.
complete+nonpreincubated S30 fraction (1 mg/ml)	1,176
complete+nonpreincubated S 30 fraction+DNAse (5 μg/ml)	1,128
complete+nonpreincubated S30 fraction+RNAse (5 μg/ml)	4
complete+preincubated S 30 fraction (1 mg/ml)	110

The reaction mixtures contained in μmole/ml: 100 Tris-HCl pH 7.8, 10 magnesium acetate, 50 KCl, 6 2-mercaptoethanol, 1 ATP, 0.03 GTP, 5 phosphoenolpyruvate, 20 μg PEP-kinase, 0.05 mixture of 20-L-amino acids minus valine, 0.018 L-valine (125 mC/mMol) and S 30 fraction. Samples were incubated at 37 °C for 25 minutes, deproteinized with 10% TCA. Precipitates were washed 3 times with 5% TCA and counted on Biospan-Nuclear Chicago counter. Background 2 imp/minute.

Fig. 9. The effect of tetracycline on the *in vitro* protein synthesis directed by endogenous mRNA.

The composition of reaction mixture was as described in Table 1.



nonpreincubated mixture contains endogenous mRNA sensitive to RNAse action. Protein synthesis in this systems is not dependent on the presence of DNA. After preincubation of the S30 fraction to destroy endogenous mRNA, synthesis of proteins was lowered to 10 % of the maximal value observed with the nonpreincubated fraction. Dialyzed S30 fraction without preincubation was used for studies of the effect of tetracycline on protein synthesis in the tetracycline-producing strain. Fig. 9 shows per cent inhibition of protein synthesis obtained at different levels of tetracycline. The results suggest that the protein-synthesizing system of *S. aureofaciens* is much more resistant to the antibiotic than a similar system isolated from sensitive bacteria. It has been reported in experiments with *E. coli*[17] that 10 μg/ml of tetracycline causes 80 % inhibition of poly U-directed synthesis of polyphenylalanine. Comparable inhibitory effect was observed in the present experiments with *S. aureofaciens* at drug concentration of at least 200 μg/ml. There is a lack of information about the localization of resistance to tetracycline in the *in vitro* protein synthesizing systems. Recently, CRAVEN *et al.*[33] isolated a tetracycline-resistant mutant of *E. coli* containing ribosomes resistant *in vitro*. Resistance to the antibiotic can be abolished or decreased by salt washing. It is not known whether this resistance is located in a ribosomal protein or in the initiation factors. More detailed informations about this problem are under examination.

The results suggest that intracellular accumulation of tetracycline during the growth of *S. aureofaciens* could lead to blockage of protein synthesis by the producing cells.

Acknowledgement

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