NUCLEIC ACID DEGRADATION PRODUCTS OF *STREPTOMYCES AUREOFACIENS*

JOZEF ŠIMÚTH and JÁN ZELINKA

Department of Biochemistry of Microorganisms,
Biological Institute, Slovak Academy of Sciences,
Bratislava, Czechoslovakia

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In industrial fermentations of *Streptomyces aureofaciens*, accumulation of hypoxanthine, cytosine and guanosine in the fermentation medium depending on the level of chlortetracycline production was observed. A similar dependance was found also in fermentation media of low- and high-producing strains of *Streptomyces aureofaciens* under laboratory conditions. Chlortetracycline supplemented to the medium for the cultivation of a low- and high-productive strains of *S. aureofaciens* in an amount of 200 and 500 μg/ml resulted in an increase of the hypoxanthine and cytosine levels. At the same time AMP and CMP in some cases were also found in the medium. Cadaverine added to the fermentation medium in an amount of 0.1 % decreased the level of cytosine by 60 % and increased the guanosine content by 50 % as compared with the control. The hypoxanthine level remained unchanged. In the acid-soluble fraction of the mycelium of *S. aureofaciens* nucleotides, nucleosides, and bases with predominance of AMP and its degradation products were found. In the fermentation medium and in the acid-soluble fraction of the mycelium, some unknown ultraviolet-absorbing substances were observed.

Studying the level of ribonucleic acid production by the chlortetracycline producer, *Streptomyces aureofaciens*, Di MARCO[5] has found that following an intense synthesis of ribonucleic acids a decrease in their content is observed towards the end of fermentation. Similar results were published by DOSKOČIL *et al.*[6] who reported that in *Streptomyces rimosus* the level of deoxyribonucleic acid does not change during fermentation while after the 10th hour of fermentation the content of ribonucleic acids suddenly begins to decrease. In our previous communication[23] we have established that in industrial fermentations of *S. aureofaciens* producing high chlortetracycline levels, even without any interference by chemical agents a more rapid decrease of ribonucleic acids in the mycelium is observed as compared with lower chlortetracycline-producing fermentations. The level of deoxyribonucleic acids does not change during the fermentation process.

On the ground of these findings we have anticipated that due to the degradation of ribonucleic acids during *S. aureofaciens* fermentations, an increase in the level of degradation products in the fermentation medium and eventually also in the acid-soluble fraction of the mycelium would take place.

Materials and Methods

Organisms. For the industrial fermentation, a high-productive strain of *S. aureofaciens* BM-K, producing 1,000~3,000 μg of chlortetracycline/ml was used. In laboratory fermentations we used also the low-producing strain, *S. aureofaciens* B96 XVI-130367 producing about 100 μg of chlortetracycline/ml.

Media and Growth Conditions. In industrial fermentations the production strain was cultivated in the following medium: corn starch 4.6 %, soy-meal 3 %, $CaCO_3$ 0.6 %, $(NH_4)_2SO_4$ 0.3 %, NaCl 0.2 %, corn steep liquor (60 % dry matter) 0.5 %, molasses 0.2 % and $CoCl_2$ 0.002 %, pH 6.8 after sterilization. Benzyl thiocyanate was added according to PECÁK *et al.*[17] at hours 0, 7, 14 and 20 in amounts of 2.5, 2.0, 1.5 and 1.5 μg/ml, respectively. The medium was aerated by 0.5 volumes of air per minute per volume of fermentation medium. In laboratory fermentations, both strains were cultivated on a rotary shaker in 500-ml flasks, containing 60 ml of fermentation medium of the following composition: sucrose 3 %, soy-meal 2 %, NaCl 0.25 %, $(NH_4)_2SO_4$ 0.2 %, $CaCO_3$ 0.4 %, molasses 0.2 %, corn-steep liquor (60 % dry matter) 0.5 %, pH adjusted to 5.8 with H_2SO_4; sterilization 1 hour at 1.1~1.2 atm.

Cadaverine in an amount of 0.1 % (v/w) was added to the fermentation medium prior to sterilization. Chlortetracycline was added aseptically in an amount of 200 or 500 μg/ml of fermentation medium in the 20 th hour of fermentation.

Analytical Methods. Fermentation medium for analytical purpose was obtained after centrifuging off the mycelium at 0°C and 3,000 r.p.m. Trichloracetic acid to make a 10 % solution was added to the supernatant. After centrifugation of the formed precipitate, the trichloracetic acid was removed by repeated extraction with ether. Then, 2/3 of the volume of the fermentation medium were evaporated on a rotary vacuum evaporator at 30°C.

The acid-soluble fraction was obtained from the centrifuged mycelium which was washed three times with a 5-fold volume of 0.9 % NaCl at 1°C. The washed mycelium was ground in a cooled grinding mortar to which acetone and solid CO_2 were added. After grinding to a fine powder, it was extracted three times for 15 minutes with 10 % $HClO_4$ at 0°C. The perchloric acid was separated by precipitation with KOH.

Substances of fatty character were removed by a 5-fold extraction with cooled ether. Afterwards the acid-soluble fraction was evaporated at 27°C in a rotary vacuum evaporator to 1/4 of its original volume.

In order to study the acid-soluble degradation products of nucleic acids in the fermentation medium and in the acid-soluble fraction of the mycelium of *Streptomyces aureofaciens*, ion-exchange column chromatography,[3,7,10] and descending paper chromatography on Whatman No. 1 paper with the following systems were used: (1) Isopropanol – conc. HCl – water (170 : 41 : 30); (2) 1-butanol – CH_3COOH – water (40 : 11 : 49); (3) 1-propanol – water (70 : 30); (4) saturated $(NH_4)_2SO_4$ solution – 1-butanol – water (75 : 5 : 19); (5) 1-propanol – 25 % NH_4OH – water (60 : 30 : 10); (6) isobutyric acid – 0.5 N NH_4OH (100 : 60); pH 3.6~3.7; (7) 96 % ethanol – 1 M CH_3COONH_4 (70 : 30). All ratios are given in volumes.

Ultraviolet spectra in the acidic and basic region after elution of the nucleotides, nucleosides and bases from paper chromatograms were measured using the recording spectrophotometer Beckman DB.

Phosphorus in the nucleotides was determined according to the method described by LOWRY and LOPEZ[13] as modified by VENKSTERN and BAEEV[22], ribose determinations were made according to MILITZER[14].

Concentrations of the identified degradation products were determined by optical density measurements at 260 mμ and comparisons made with standards (Koch-Light) of known concentrations. In the fermentation medium no measurable amounts of nucleic acid degradation products were found. A detailed description of the methods used is published elsewhere[19].

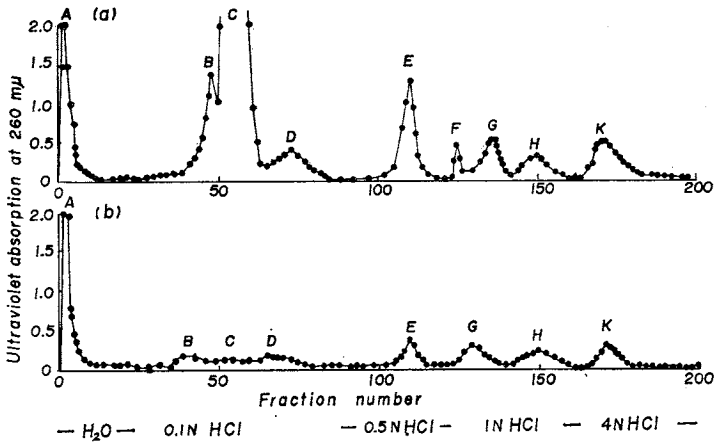
Results

The degradation products of ribonucleic acids in the fermentation medium of *S. aureofaciens* were studied in industrial fermentations in batches with different chlortetracycline production. Fig. 1 shows the results of ion-exchange column chromatography of nucleosides and bases from fermentation media of two industrial fermentations with different production of chlortetracycline at the 40 th hour of fermentation.

In the fermentation medium hypoxanthine, guanosine, and cytosine were found. Exceptionally we observed also AMP and CMP in an amount of approximatively

Fig. 1. Ion-exchange column chromatography of nucleotides and bases.
25 ml of fermentation medium; column 1.2×20 cm, Dowex 50 W×8, 200/400 mesh; flow rate 0.4 ml/min.; 4 ml fractions. Eluent: hydrochloric acid from 0.1 to 4.0 N.

(a)—fermentation with a yield of 2,960 μg/ml of chlortetracycline
(b)—fermentation with a yield of 1,060 μg/ml of chlortetracycline



A—yellow-brown substance; B,C,D—unidentified UV-absorbing substances; E—hypoxanthine; F—unknown spot, green in UV-light; H—guanosine; G—cytosine; K—chlortetracycline.

Fig. 2. Hypoxanthine, cytosine, and guanosine levels in the fermentation medium and the production of chlortetracycline during industrial fermentation.

(a)—fermentation with a yield of 2,960 μg/ml of chlortetracycline
(b)—fermentation with a yield of 1,060 μg/ml of chlortetracycline

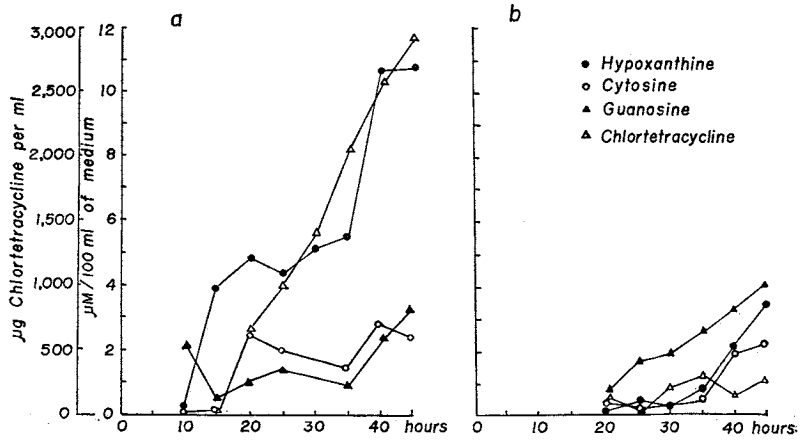


Fig. 3. Spectrum of the unidentified substance from fractions B, C, D (Fig. 1) in 0.1 N HCl after elution from paper chromatogram.

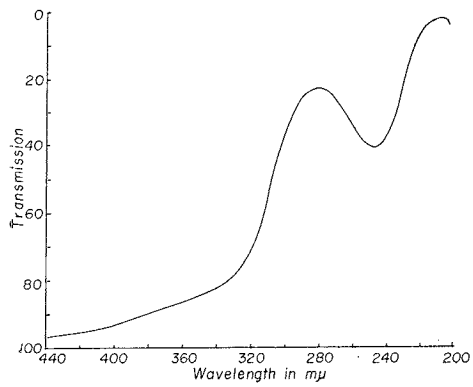
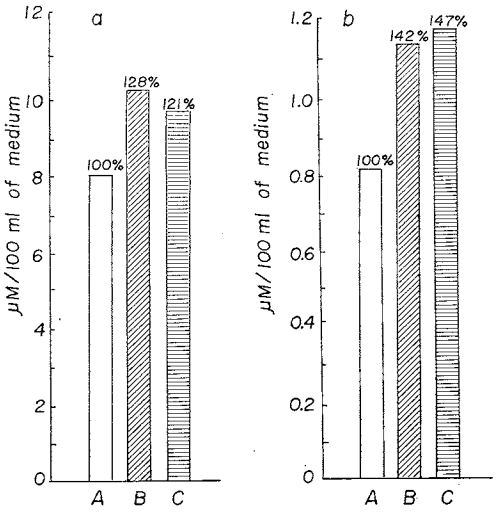


Fig. 4. Hypoxanthine content in medium at the 72 nd hour of laboratory fementation.

(a)—high-producing strain of *S. aureofaciens*

(b)—low-producing strain of *S. aureofaciens*



A—Control

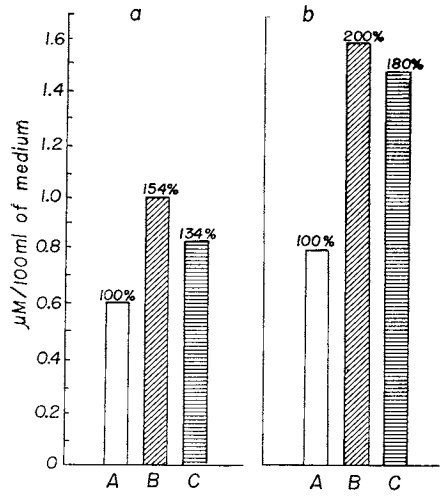
B—After addition of 200 μg of chlortetracycline/ml of fermentation medium

C—After addition of 500 μg of chlortetracycline/ml of fermentation medium

Fig. 5. Cytosine content in medium at the 72 nd hour of laboratory fermentation

(a)—high-producing strain

(b)—low-producing strain

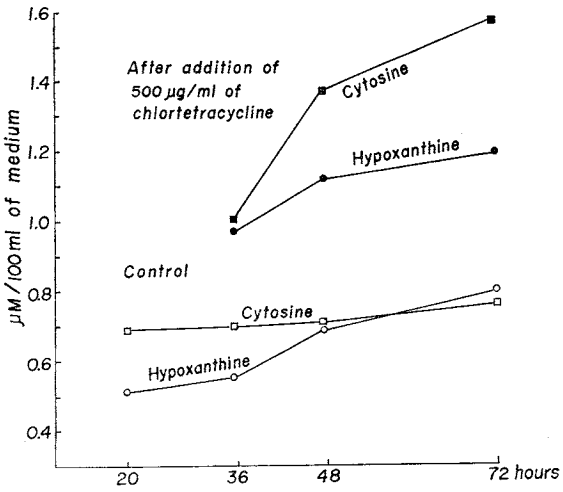


A—Control

B—After addition of 200 μg of chlortetracycline/ml of fermentation medium

C—After addition of 500 μg of chlortetracycline/ml of fermentation medium

Fig. 6. Hypoxanthine and cytosine level in fermentation medium of low-producing strain of *Streptomyces aureofaciens* during laboratory fermentation.



1 μM/100 ml of medium. The levels of hypoxanthine, cytosine and guanosine in the fermentation medium as well as the production of chlortetracycline during fermentation are shown in Fig. 2.

In fractions B, C, D (Fig. 1) there are ultraviolet-absorbing substances not show-

ing paper chromatographic and ultraviolet-spectrophotometric properties of known purine and pyrimidine compounds or their derivatives. They show characteristic ultraviolet spectra with a maximum at 280 mμ and minimum at about 240 mμ. In Fig. 3 the spectrum of the prevailing substance eluted from the paper chromatogram as measured in 0.1 N HCl is presented. With the continuing biosynthesis of chlortetracycline, the content of these unknown substances in the fermentation medium gradually increases.

We have further compared the content of degradation products of nucleic acids in a fermentation medium of a low- and high-productive strain cultivated under laboratory conditions. We have found that under laboratory and industrial conditions identical substances can be identified. Similarly as in industrial fermentations, apart from the exceptional occurrence of CMP and AMP we have not determined any other nucleotides. Among nucleosides we have found guanosine and from bases, cytosine and hypoxanthine. In the fermentation medium of the low-productive strain the content of hypoxanthine was ten times lower as compared with the high productive strain, while the cytosine content was approximatively equal in both strains. In the fermentation medium of the low-productive strain only traces of guanosine were found.

Further we studied the effect of added chlortetracycline on the level of degradation products of ribonucleic acids in the fermentation medium of *Streptomyces aureofaciens* during laboratory fermentations. The increase of the hypoxanthine and cytosine levels in the fermentation medium at the 72 nd hour of laboratory fermentation of the studied strains is presented in Fig. 4 and Fig. 5. The added chlortetracycline caused only an increase in hypoxanthine and cytosine, while did not affect the level of guanosine. The comparison of the hypoxanthine and cytosine levels in the control and after addition of chlortetracycline to the low-productive strain is shown in Fig. 6.

On ground of our other observation[25] on the effect of added cadaverine on the level of nucleic acids in *S. aureofaciens* during fermentation, we have considered it necessary to examine its effect on the accumulation of degradation products of nucleic acids in the fermention medium of the high-productive strain. We have found that the level of hypoxanthine does not change under the influence of cadaverine, while the guanosine content as compared with the control increases by about 50 %. Due to the effect of cadaverine the content of cytosine decreases by 60 %.

At 20, 48 and 72 hours of the fermentation degradation products of the nucleic acids in the acid-soluble fraction of the mycelium of the high- and low-productive strains of *S. aureofaciens* were determined. Nucleotides, nucleosides, and hypoxanthine were found in the acid-soluble fraction; the results are listed in Table 1. Apart from the substances reported in the Table we have found also some unknown substances observed in high amount also in the fermentation medium. The main part of the ultraviolet-absorbing substances contained in the acid-soluble fraction is constituted by an unidentified brown-yellow substance which remains on a column of Dowex 50W H^+ even after elution with 4 N HCl.

Table 1. Content of nucleic acid degradation products in the acid-soluble fraction of the mycelium of *Streptomyces aureofaciens*.

Hours of fermentation	Strain	μM/100 g of centrifuged mycelium						
		AMP	CMP	UMP	Adenosine	Guanosine	Inosine	Hypoxanthine
20	high-productive	5.7	1.1	1.7	5.3	0.9	—	0.34
	low-productive	1.7	—	—	2.9	0.5	3.2	0.23
48	high-productive	—	—	—	—	—	—	0.8
	low-productive	2.55	—	—	6.8	—	—	0.30
72	high-productive	—	—	—	5.2	—	—	—
	low-productive	2.1	—	—	4.9	—	—	0.18

Discussion

Fig. 1 and Fig. 2 indicate that nearly simultaneously with the biosynthesis of chlortetracycline hypoxanthine appears in the fermentation medium and its level increases in accordance with chlortetracycline production. The guanosine and cytosine contents do not show such a distinct relationship to chlortetracycline biosynthesis as does hypoxanthine. The guanosine and cytosine contents in high-productive fermentation is as much as four times lower than the content of hypoxanthine. As a possible degradation product of RNA the surplus of hypoxanthine as compared with cytosine and guanosine is only apparent. Taking into account the results of ZELINKA and SCHNITTOVÁ[23], in reality the hypoxanthine content in the fermentation medium corresponds with the expected AMP content in that part of RNA by which its content was decreased in the mycelium. Although an accumulation of nucleotides as a result of enzymatic degradation of intracellular RNA is often observed in microorganisms,[9,16,18] we have observed only a sporadic occurrence of AMP and CMP in the fermentation medium. Similarly we have found a relatively low content of nucleotide-mono-phosphates in the acid-soluble fraction of the mycelium (Table 1). Among the substances found, AMP as determined in *S. rimosus* also by GUBERNIEV, UGOLEVA and TORBOCHKINA[8] prevails. This discrepancy between our findings and the results known from literature can, to a certain degree, be explained by the study of IMADA, MITZUO and SEIZI[11] who found an intracellular enzyme hydrolyzing the N-glycosidic linkage in 5'-nucleotides in various Streptomycetes, the enzyme having different activities towards individual nucleotides. A different enzymatic activity of enzymes decomposing nucleotides was observed also in other microorganisms.[12,20] According to DEMAIN and HENDLIN[4] the adenine-dependent mutant of *Bacillus subtilis* accumulates hypoxanthine and inosine in the fermentation medium. These authors did not observe the accumulation of the respective 5'-IMP, but they isolated the appropriate 5'-nucleotidase. CHIROSI, TAKASI and ISMIANA[2] reported the production of enzymes degradating 5'-AMP to adenosine, adenine, and inosine by a Streptomycete. The results show that in the metabolism of *S. aureofaciens* enzymes take part, which decompose nucleotides to lower degradation products at different rates.

It is generally known that penicillin,[21] streptomycin[1] and decoine[15] addded to growing cultures, cause an increased accumulation of nucleotides and of their components. The accumulation of RNA degradation products takes place even when a lack of nutrients is observed. In *E. coli* MARUYAMA and MIZUNO[13a] described an intense degradation of ribosomal RNA and accumulation of its degradation products after the phosphorus of the medium was used up. In our experiments all nutrients were maintained in a sufficient level during the whole fermentation both in the control and in the experimental flasks to which chlortetracycline was added.

Chlortetracycline added in an amount of 200 or 500 μg/ml at the beginning of the production-phase of the antibiotic (Figs. 4, 5, 6), caused an increase in the hypoxanthine and cytosine levels in the medium of the producing strain, too. The exogenous chlortetra-

cycline did not affect the content of guanosine and of other possible degradation products of nucleic acids, *i. e.* the added chlortetracycline affects the level of the studied metabolites in a similar way as that synthesized by the producer. This corroborates our hypothesis[24] that the produced chlortetracycline in itself affects the degradation of ribonucleic acids of the producing strain.

According to ZILLIG, KRONE and ALBERS[26] diamines present in ribosomes are strong inhibitors of ribonuclease. We have found[25] that cadaverine influences the level of ribonucleic acids in the mycelium of the high-producing strain of *S. aureofaciens* and we therefore anticipate that cadaverine protects the structure of the ribosomes of the producing strain against the effect of the chlortetracycline produced. The results suggest that apart from the stabilizing effect of cadaverine on ribosomes of the producer, it affects also the enzymatic system which causes nucleotide degradation.

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