THE BIOSYNTHESIS OF TETRACYCLINE

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ABSTRACT

It was initially assumed that the tetracycline nucleus originated by the polyketide pathway from acetyl- or malonyl-CoA. Evaluation of the results of experiments with labelled precursors and on the regulation of presumed key enzyme systems, however, has led to the conclusion that the situation is more complex than was first thought. It is suggested that chlortetracycline does not fit into the category of the so-called secondary metabolites and should be regarded rather as an 'excessive metabolite' produced by variation in the control of certain key primary pathways. The experimental evidence on control of pathways during the various phases of the bacterial growth cycle is reviewed.

INTRODUCTION

Abundant experimental evidence has accumulated over the past years that the relatively large group of natural compounds designated as oligoketides are formed by condensation of acetate and malonate units. A detailed description of the history of research into this metabolic pathway may be found in reviews and monographs[1–4].

Nevertheless, even a short review of the concepts relevant to the biosynthesis of cyclic and aromatic compounds[5]—the older view of condensation of hydroxyphthalic acids with phenolcarboxylic acids[6–8], or the statistical approach assuming 24 possible combinations of four acetate units during biosynthesis of orsellinic acid and more than 40000 combinations during biosynthesis of compounds arising by condensation of eight acetate units[9]; the view of a supplementary oxidation of preformed anthraquinones which aids in elucidating the synthesis of compounds with branched chains[10] and the closely connected role of a hypothetical orsellinyltriacetic acid[11]; consideration of a possible multi-enzyme complex and enzyme reactions taking place on its level[12]; the concept of biosynthesis of more complicated oligoketides through condensation of short incomplete chains, e.g. $C_8 + C_6$, where changes like methylation, reduction, keto–enol tautomerism take place before condensation and can decide the type of compounds that will be produced by the microorganism under certain environmental conditions[13]—shows that much remains to be solved even at the level of the principal building blocks. Our present knowledge in this field could not yet be applied to the treatment of systems controlling the biosynthesis of oligoketide-type compounds.

All papers stress the similarity with the biosynthesis of fatty acids with the sole difference that intermediate polyketothioesters remain completely or partly in the oxidized form during condensation. Experiments with incorporation of labelled acetyl or malonyl thioesters in cell-free extracts into 6-methylsalicylic acid[14], patulin[15], stipitatic acid[16], and alternariol[17] were in agreement with this basic view.

Some discord was introduced into the harmony by work on the tetracycline series. Although the view is generally accepted that the terminal group of the hypothetical nonaketide is formed by malonamyl-CoA[18–21], some papers indicated[22–23] that not all the problems by far of the biosynthesis of the tetracene skeleton have been satisfactorily answered. The existing modes attempts at understanding the regulation of the biosynthesis of these compounds on the enzyme level suggest that the formation of chlortetracycline in high-production strains of *Streptomyces aureofaciens* cannot be accounted for simply by an intensive carboxylation of acetyl-CoA to malonyl-CoA. Thus, under industrial fermentation conditions when the medium contains a great deal of protein, one must also take into account other precursors or metabolic pathways that might lead to the formation of malonyl-CoA.

EXPERIMENTS WITH LABELLED PRECURSORS

Evaluation of the results of the first paper[24] dealing with the biosynthesis of chlortetracycline with the aid of radioactive precursors lay predominantly in a comparison of the specific radioactivities of chromatographically isolated chlortetracycline after adding some 40 different labelled compounds into the cultivation medium (a degradation procedure was used for the localization of radioactivity after incorporation of 2-^{14}C-glycine and $^{14}CH_3$-methionine into the dimethylamino group of ring A of the chlortetracycline molecule). It was found that 80–90 per cent chlortetracycline is formed from starch and, furthermore, that the metabolic pathway for the biosynthesis of chlortetracycline does not involve fructose, pentoses, the Krebs cycle, shikimic acid and fixation of carbon dioxide. It could be concluded on the basis of this work that chlortetracycline is a representative of oligoketide compounds.

More recent work using labelled substrates supplemented and confirmed these first data. The question of the Krebs cycle remained open, particularly from the point of view of control of chlortetracycline biosynthesis which will require further investigation.

Chemical degradation of labelled oxytetracycline showed that the radioactivity of the ring D carbon atoms differs from the radioactivity of the ring A carbon atoms of the oxytetracycline molecule. Certain differences in the results may be ascribed to the time (physiological state of the culture) when the precursor is added to the cultivation medium and to the duration of incubation with the radioactive precursor[25]. A more important finding appears to be the relatively high degree of randomization of radioactivity after addition of $^{14}CH_3COOH$ among carbon atoms which, in the tetracene skeleton, correspond to carbonyl carbons according to the oligoketide rule[26]. The relatively high degree of randomization (1/6) indicates that acetic

acid is not incorporated directly into the tetracycline molecule (acetyl-CoA → malonyl-CoA → tetracycline) but that it first passes through the metabolic cycles of dicarboxylic or tricarboxylic acids. For comparison, randomization accounts for only 2 per cent of total radioactivity of 2-^{14}C-acetic acid incorporated into 6-methylsalicyclic acid of *Penicillium griseofulvum*[27].

In this connection, it is of interest to note the results obtained with the biosynthesis of 6-methylsalicyclic acid formed by condensation of one molecule of acetyl-CoA and three molecules of malonyl-CoA in *Mycobacterium phlei*. The incorporation of 2-^{14}C-acetic acid into this compound was very low in this case[28]. Incorporation amounted here to thousandths or hundredths of one per cent while incorporation of the same compound into fatty acids under the same conditions amounted to almost 10 per cent. This value is of importance as it shows that the low incorporation of acetic acid into 6-methylsalicyclic acid is not due to low permeability of the cell membrane with respect to this compound. Roughly similar incorporation as with 6-methylsalicyclic acid was found in phenylacetate and tyrosine which are generally believed to be formed by the shikimate pathway. Chemical degradation of labelled tyrosine then showed that 67.4 per cent of the total radioactivity resided in the aromatic ring.

The difficulty of the evaluation of the incorporation experiments and the complexity of the metabolic relations existing in the living organism emerge from the example of chartreusin, a glycoside produced by a *Streptomyces* sp., the aglycone of which is formed according to the acetate–malonate rule[29]. Acetic acid (in contrast with diethyl malonate) is again scarcely incorporated into the aglycone. A substantially greater incorporation was achieved with 1-^{14}C-*trans*-cinnamic acid while the incorporation of 3-^{14}C-*trans*-cinnamic acid was negligible. Apparently, 1-^{14}C-cinnamic acid is β-oxidized to radioactive acetyl-CoA which is utilized for the biosynthesis of the oligoketide skeleton of the aglycone of the chartreusin molecule.

Experiments with labelled acetic acid or malonic acid (or with the esters) can hardly provide new information or a deeper insight into the mechanisms underlying the biosynthesis of tetracycline in our case. What we lack is above all experimental data on the precursors and enzyme systems from which acetyl-CoA or malonyl-CoA are formed whilst the microorganism does not grow further and catabolic reactions predominate. In a situation when the cell has ceased to grow, when proteins are not synthesized and cell walls are not enlarged, one can image that a shift has taken place in the metabolic pathways contributing during the different periods to the common pool of acetyl- or malonyl-CoA.

PYRUVATE AS A SOURCE OF ACETYL-CoA AND MALONYL-CoA

Investigation of metabolic pathways in *Streptomycetes* has shown that organic acids accumulate in the course of cultivation of these microorganisms[30]. We are chiefly interested in pyruvic acid[31] and lactic acid[32] since their accumulation signals that oxidative decarboxylation of pyruvic acid to acetyl-CoA as the basic building unit of oligoketides has been

restricted. To increase the concentration of pyruvic acid one may cultivate streptomycetes in synthetic media. This suggests that pyruvate dehydrogenase is an enzyme complex, sensitively responding to the conditions of the external medium (in a broad sense of the word).

When studying the biosynthesis of chlortetracycline in *S. aureofaciens* under production conditions, pyruvic acid was detected (*Figure 1*) about the

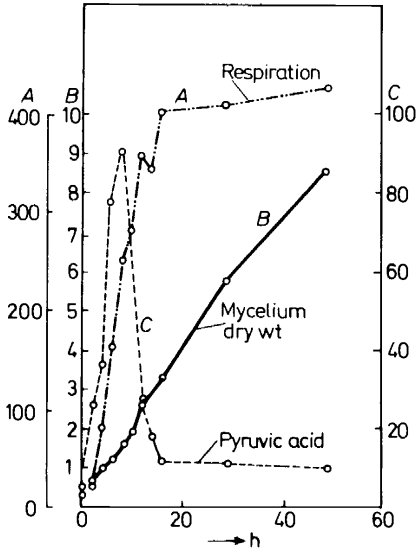


Figure 1. Growth, respiration and pyruvic acid level during the cultivation of *Streptomyces aureofaciens* in shaken flasks. A, respiration (ml O_2 l^{-1}, culture h^{-1}); B, dry wt. mycelium (mg ml^{-1}); C, pyruvic acid (μg ml^{-1}) From *J. Biochem. Microb. Technol. Eng.* **1**, 261 (1959)

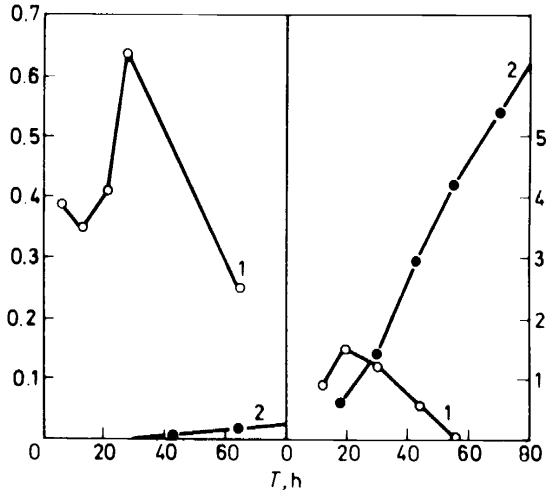


Figure 2. Specific activity of pyruvate dehydrogenase complex in the course of cultivation of low-producing (left) and producing (right) strains of *S. aureofaciens* 1—spec. activity (μmols ferrocyanide per 30 minutes per mg protein); 2—chlortetracycline (μg ml^{-1} × 10^{-3})

12th hour of cultivation (about 100 μg ml^{-1}). During subsequent hours of cultivation, the content of this acid in the cultivation medium drops sharply[33]. The transient increase of pyruvate concentration in the cultivation medium of *S. aureofaciens* is caused by the inability to remove phosphoenol-pyruvate[34, 35] through the tricarboxylic acid cycle. This observation agrees with the examination of the activity of the pyruvate dehydrogenase complex in the course of cultivation of *S. aureofaciens*. The maximum activity of this enzyme in the production strain appeared between 12 and 24 hours of cultivation (*Figure 2*). After 48 hours, at the time of a sharp increase of the enzyme systems synthesizing chlortetracycline, the activity of the dehydrogenase system is very low. Similar results were obtained when estimating the activity of acetyl-CoA carboxylase. With this enzyme, too, the activity during the production period is very low even if during the 12th and 24th hours of fermentation it is clearly higher in the high-production strain of *S. aureofaciens* than in the low-production one (*Figure 3*). It is thus thought

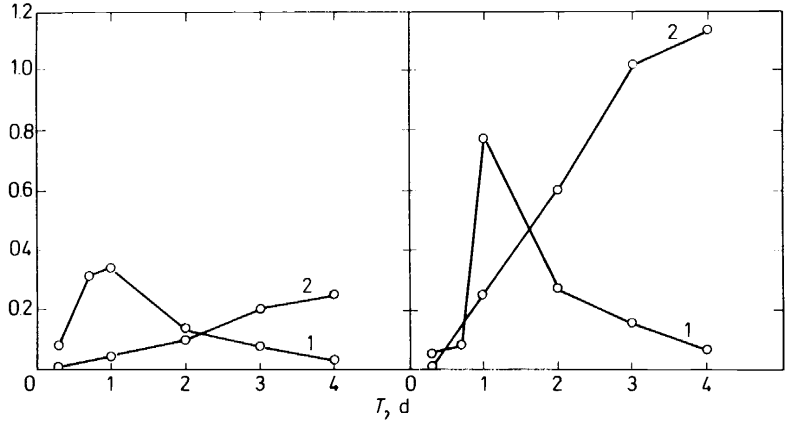


Figure 3. Specific activity of acetyl-CoA carboxylase in the course of cultivation of low-producing (left) and producing (right) strain of *S. aureofaciens*. 1—spec. activity (c.p.m. mg^{-1} protein × 10^{-4}); 2—chlortetracycline (μg ml^{-1} × 10^{-3})

that the decrease of enzyme activities, including those of the tricarboxylic acid cycle at about 24 hours of fermentation[36, 37], is in a first approximation due to restriction of protein synthesis. Glutamic and aspartic acids, as well as other amino acids associated with the tricarboxylic acid, are not formed. In this situation it may happen that the concentration of some organic acids of the tricarboxylic acid cycle will be increased. During the subsequent hours of cultivation, the cell compensates for this increase by opening new metabolic relations in which catabolic reactions predominate.

A drop in pyruvic acid may occur either in consequence of induced synthesis of the dehydrogenase enzyme complex or due to removal of an inhibitor, e.g. succinate (it should be noted in this context that the cultivation medium of *S. rimosus* was found to contain large amounts of α,β-diamino-succinic acid[38]). Another possibility would be the carboxylation of pyruvic

acid to oxaloacetic acid (using the biotin enzyme plus ATP) or carboxylation to malic acid by reductive carboxylation with NADPH[39]. In our experiments, we could not demonstrate pyruvate carboxylase that could give rise to oxaloacetic acid. It may be concluded from the enzyme activity of malate dehydrogenase (decarboxylating) that the enzyme does not participate in chlortetracycline biosynthesis (*Figure 4*).

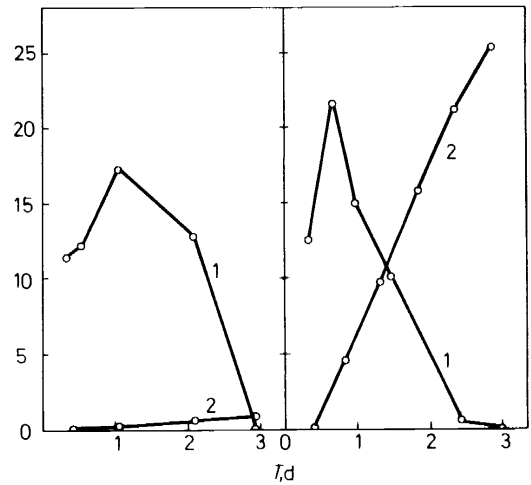


Figure 4. Specific activity of malate dehydrogenase (decarboxylating) in the course of cultivation of low-producing (left) and producing (right) strains of *S. aureofaciens* 1—specific activity (μmol μg^{-1} protein min^{-1}); 2—chlortetracycline (μg $ml^{-1} \times 10^{-3}$)

Accumulation of α-keto acids was also observed in *S. rimosus* (a producer of oxytetracycline[40]). It was found that pyruvic acid is converted stoichiometrically to α-ketoglutaric acid.

It was said before that the oxidation of pyruvic acid to acetyl-CoA as the single metabolic pathway yielding the basic building blocks of chlortetracycline is contradicted by the results obtained with labelled precursors. It must be borne in mind, however, that addition of acetic acid to the medium does not correspond to physiological conditions. To convert labelled acetic acid added to the medium to acetyl-CoA, the cell requires considerable energy. This can be drawn from the cleavage of ATP (this gives rise to adenylic anhydride as an intermediate product) or from a markedly exergonic reaction, such as oxidative decarboxylation. It was found in the present experiments that the concentration of ATP in the low-production strain shows two peaks and is about 10 times greater than in the high-production strain. The ATPase activities are about equal. In the low-production strain, the maximum is reached after some 36 hours of cultivation, in the high-production strain after some 48 hours (*Figure 5*). When studying the incorporation of 1-^{14}C-acetic acid in high- and low-production strains of *S. aureofaciens* into fatty acids it was observed that the activity of the enzyme system is about 5 times greater in the high-production strain and attains

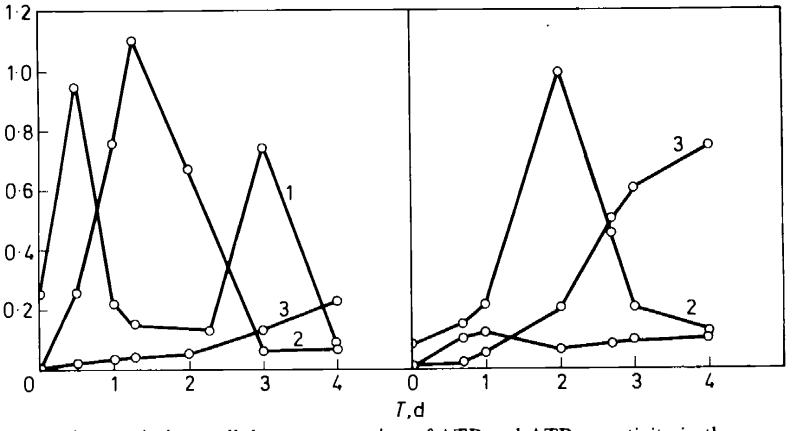


Figure 5. Changes in intracellular concentration of ATP and ATPase activity in the course of cultivation of low-producing (left) and producing (right) strains of *S. aureofaciens* 1—ATP (μg ml^{-1}); 2—ATPase (orthophosphate μg ml^{-1} protein × 5.10^{-3}); 3—chlortetracycline (μg ml^{-1} × 5.10^{-4})

maximum production between 20 and 24 hours of cultivation (*Figure 6*). These results, like the randomization of radioactivity after adding 2-^{14}C-acetic acid, indicate that the carboxylation of acetyl-CoA to malonyl-CoA

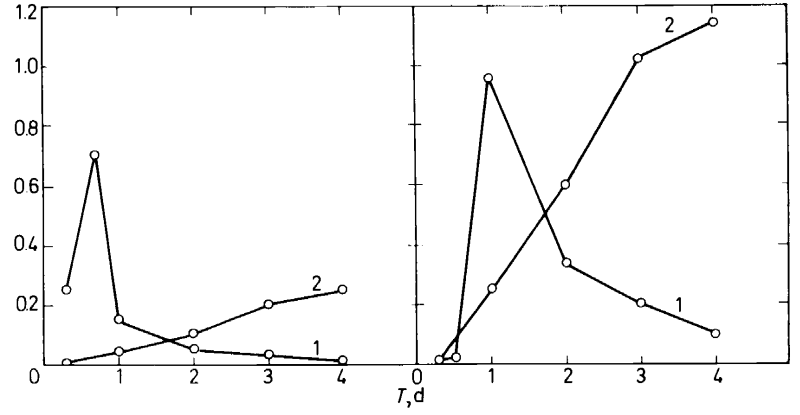


Figure 6. Incorporation of 1-^{14}C-acetic acid into fatty acids during cultivation of low-producing (left) and producing (right) strain of *S. aureofaciens* 1—incorporation of labelled acetate (μCi g^{-1} mycelial dry weight); 2—chlortetracycline production (μg ml^{-1} × 10^{-3})

is not the sole source of the building units of the tetracene skeleton. It appears that at least a part of acetyl-CoA is drawn into the dicarboxylic acid pool. It is thus likely that the carboxylation of acetyl-CoA to malonyl-CoA is the bottleneck of chlortetracycline synthesis.

OTHER PRECURSORS OF MALONYL-CoA

The literature contains ample data that malonyl-CoA need not arise merely by carboxylation of acetyl-CoA. A number of other reactions were

described, all of them giving rise to malonic acid or rather to malonyl-CoA. For example, malonyl-semialdehyde was found to be formed in rat kidney where it is the product of transamination of β-alanine with 2-oxoglutarate. Similarly, malonyl-semialdehyde was detected in a partially purified enzyme preparation from a *Pseudomonas* species (transamination between β-alanine and pyruvate). The β-alanine considered in these reactions is formed during metabolism of dihydrouracil or of propionyl-CoA. Enzyme-bound malonyl-semialdehyde is further assumed to be the intermediate during conversion of β-alanine to 3-hydroxypropionate in *Clostridium propionicum*[41]. Malonyl-CoA was also described as a product of oxidation of propionic acid in *Clostridium cluyveri*. Culture extracts of this microorganism catalyze reversible hydration of acrylyl-CoA to 3-hydroxypropionyl-CoA. Malonic acid can be further formed as a product of bacterial degradation of pyrimidines[42].

Malonic acid was identified, as the main organic acid, in bean roots (*Phaseolus vulgaris*) where the acetyl-CoA carboxylase activity in root tissue sections was very low (this contrasts with the embryonic tissue). It was shown in further work that the immediate precursor of malonic acid in the roots of this plant is oxaloacetic acid[43–45]. Investigation of the enzyme catalyzing the oxidative decarboxylation of oxaloacetate showed it to be probably a peroxidase.

Another possibility of formation of malonyl-CoA lies in the hypothetical transcarboxylation reactions[46–48] which might play a role in the biosynthesis of oligoketides since they have very small energy requirements.

For the sake of completeness, let me mention that during degradation of aminoadipic acid, glutaric acid is formed after transamination and oxidative decarboxylation. After attaching coenzyme A, the glutaric acid is further degraded as a fatty acid. This means that an intermediate to be considered here is the unsaturated glutaconic acid which gives rise to 3-hydroxy- and further to 3-keto-glutaric acid which is split to acetyl-CoA and malonyl-CoA[49].

The possibility of the condensation of two molecules of malonyl-CoA that might then be decarboxylated to 3-ketoglutaric acid (or the simple condensation of acetyl-CoA with malonyl-CoA) and subsequent metabolism as described above to 2-ketoglutaric acid, is not found in the literature. It would represent a fine bridge to the tricarboxylic acid cycle.

Compounds which are formed by condensation of acetyl-CoA and two molecules of malonyl-CoA, i.e. triacetic acid (3,5-dioxohexanoic acid) or its lactone (4-hydroxy-6-methyl(2H)-pyran-2-one) were described as metabolites formed in the absence of NADPH in pigeon liver[50], in *Escherichia coli*[51] and in *Penicillium patulum*[52, 53]. Derivatives of triacetic acid lactone (4-hydroxy-6-(2-oxopropyl(2H))-pyran-2-one and 3,6-dimethyl-4-hydroxy-(2H)-pyran-2-one) were isolated from the cultivation medium of *Penicillium stipitatum*[54–58].

THE TERMINAL GROUP

It was mentioned before that the terminal group of the chlortetracycline molecule is formed by malonamyl-CoA[18]. This view is indirectly supported

by isolation of 2-acetyl-decarboxamidotetracycline[59] where the terminal group is formed by acetoacetyl-CoA. Fatty acid analysis of the mycelium[60-62] of *Streptomyces aureofaciens* showed that the main component is formed by branched fatty acids, viz. 13-methyltetradecanoic, 14-methylpentadecanoic and 14-methylhexadecanoic. It is without doubt that the terminal group of these fatty acids arises from the corresponding branched amino acids (after deamination and decarboxylation during condensation with malonyl-CoA shorter by one carbon atom—*Table 1*).

Table 1. Representation of fatty acids in the lipid fractions of *Streptomyces aureofaciens*

Acid	Relative elution volume of Me ester[a]	Relative %			
		Triglycerides	Diglycerides	Free fatty acids	Mycelium hydrolyzate[b]
Lauric acid, $C_{12:0}$	0.29	0.4	0.4	0.4	0.4
Myristic acid, $C_{14:0}$	0.37	2.0	0.8	1.6	0.9
12-Methyltridecanoic acid, $iC_{14:0}$	0.47	2.3	1.2	1.9	2.9
13-Methyltetradecanoic acid, $iC_{15:0}$	0.55	17.6	22.0	26.0	23.2
14-Methylpentadecanoic acid, $iC_{16:0}$	0.76	12.1	14.3	15.6	19.2
Palmitic acid, $C_{16:0}$	1.00	15.4	15.9	14.9	17.4
Palmitoleic acid, $C_{16:1}$	1.02	12.9	11.3	14.8	0
14-Methylhexadecanoic acid, $aC_{17:0}$	1.16	14.2	15.5	15.9	27.8
Stearic acid, $C_{18:0}$	1.78	2.7	2.0	1.8	0
Oleic acid, $C_{18:1}$	1.94	9.8	6.1	4.9	0
Linoleic acid, $C_{18:2}$	2.30	9.6	7.1	4.3	0

[a] Vg (methylpalmitate) = 218.3 ml.
[b] Fatty acids remaining in the mycelium after extraction and liberated by alkaline hydrolysis.

One might visualize that the terminal group of the hypothetical nonaketide molecule of tetracycline is also formed by an amino acid, unbranched in this case, from which several metabolic reactions lead to malonamyl-CoA. This view was supported by the present results with labelled asparagine.

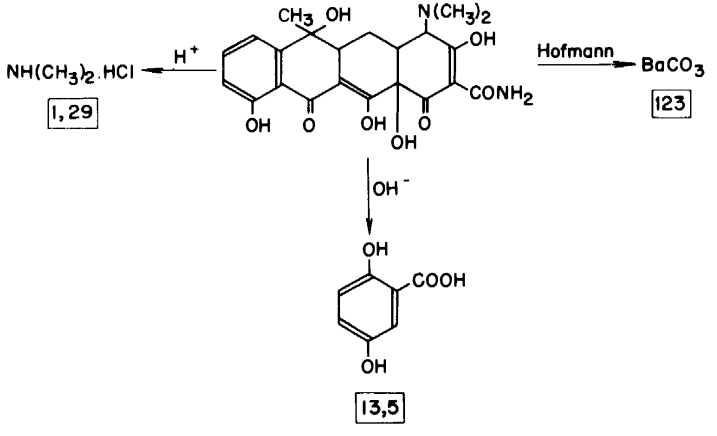
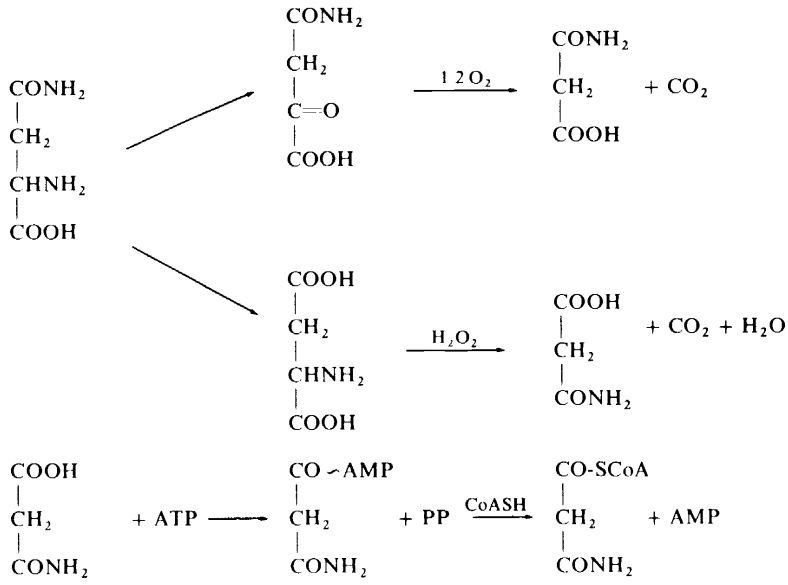


Figure 7. Scheme of tetracycline degradation and the values of the radioactivity (r.m.a. × 10^{-3}/1 C)

Degradation experiments showed[63] that the radioactivity in the chlortetracycline molecule is distributed unevenly. The radioactivity of the amide carbon arising from the assumed terminal group was about ten times greater than the radioactivity of carbons in the C and D rings of the chlortetracycline molecule (measured as gentisic acid or 6-acetylsalicyclic acid) (*Figure 7*).

As an approximation, one can imagine two paths for the transformation of asparagine to malonamide (*Scheme 1*). In the first case, the amide group of asparagine is retained during the formation of malonamide. In the second case, the amino nitrogen of asparagine becomes the donor of the amide



Scheme 1. Transformations of Asparagine to malonamide.

group of malonamide. This can be activated by ATP to malonamyl adenylate or to malonamyl-CoA. In connection with the formation of amides one may assume that amino acids and pyridoxal phosphate first react to form the corresponding Schiff's bases[64]. Peroxidase forms with the base a strong electrophilic complex. After decarboxylation the complex is hydrolyzed, the hydrolysis product being an amide.

THE EFFECT OF ANTIBIOTICS

The effect of antibiotics on their producer has been studied relatively frequently[65-67] even if not at the enzyme level. In most cases, the selective effect on low-producing strains was examined[68-71]. It was generally assumed that the high concentrations of compounds added to the cultivation medium can be tolerated only by the high-production mutants. The mechanism of action of the antibiotic in these experiments cannot be elucidated satisfactorily. A major role is played by the external medium. A production

strain of *Streptomyces griseus* (2 mg streptomycin ml^{-1}) grew well during cultivation on a solid production medium and tolerated relatively high concentrations of streptomycin added to the medium at the beginning of cultivation. During cultivation on a non-production medium (potato agar) it was sensitive to streptomycin just as were other control low-production strains.

Work dealing with the biosynthesis of chloramphenicol in *Streptomyces venezuelae* showed the existence of two mechanisms at the metabolic level which control the production of the antibiotic[72]. When adding different amounts of the antibiotic at the beginning of cultivation (lower, equal and higher amount of chloramphenicol than was produced by the cells in the given medium) the overall concentration of the antibiotic was about the same at the end of fermentation. It is highly likely that at the higher concentrations of the antibiotic, enzyme systems were induced to degrade chloramphenicol actively to biologically inefficient compounds[73]. It was demonstrated that a culture of *Streptomyces* sp. 3022a degrades the synthesized chloramphenicol to *p*-nitrobenzyl alcohol and to *p*-nitrobenzoic acid. The main degradation product was *N*-acetyl-*p*-nitrophenylserinol[74].

Evaluation of the data published on the effect of tetracycline antibiotics on their producer is very complicated. The application of terms like high-

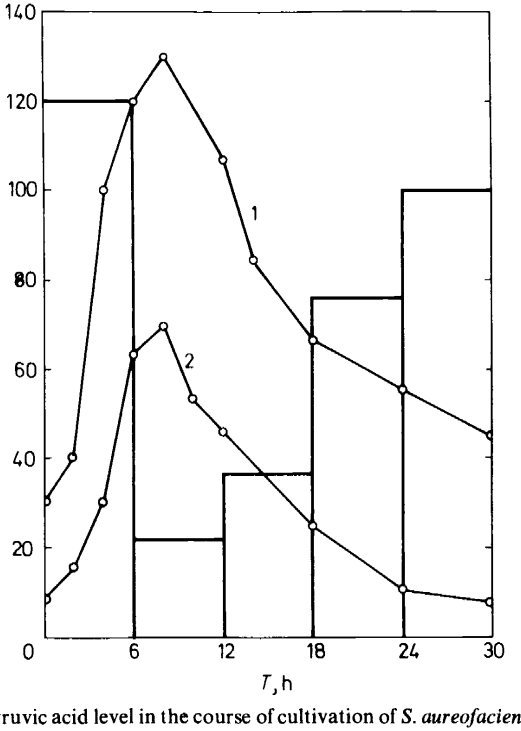


Figure 8. Q_{O_2} and pyruvic acid level in the course of cultivation of *S. aureofaciens* Bg. The column graph shows the effect of aeration interruptions of the final production of CTC as percentage of control culture yields. The aeration was interrupted every hour for 10 min, i.e. in the first group between start and 6th hour, in the second group, between 6th and 12th hour, etc.

production and low-production strains is not done rigorously in the literature. In industrial practice (strains of *Streptomyces aureofaciens* producing mg of chlortetracycline per ml after 72 or 96 hours of cultivation) a production below 500 μg ml^{-1} cultivation medium is disregarded and such strains are designated as non-producing.

Also in the case of tetracycline antibiotics it was confirmed that addition of the antibiotics at the beginning of cultivation (or even to the sporulation media) has a favourable selection effect on the antibiotic production. Experimental results showed that the young mycelium just beginning to grow is particularly sensitive to external effects[75]. A brief interruption of aeration between the 6th and the 12th hours of cultivation (*Figure 8*) has a drastic effect on the production of the antibiotic even if other metabolic processes (increase of bio-mass) remained intact[76,77].

After adding 500 μg TC ml^{-1} cultivation medium to four-hour cultures (*Figure 9*), a TC-producing strain of *S. aureofaciens* exhibited a 37 per cent

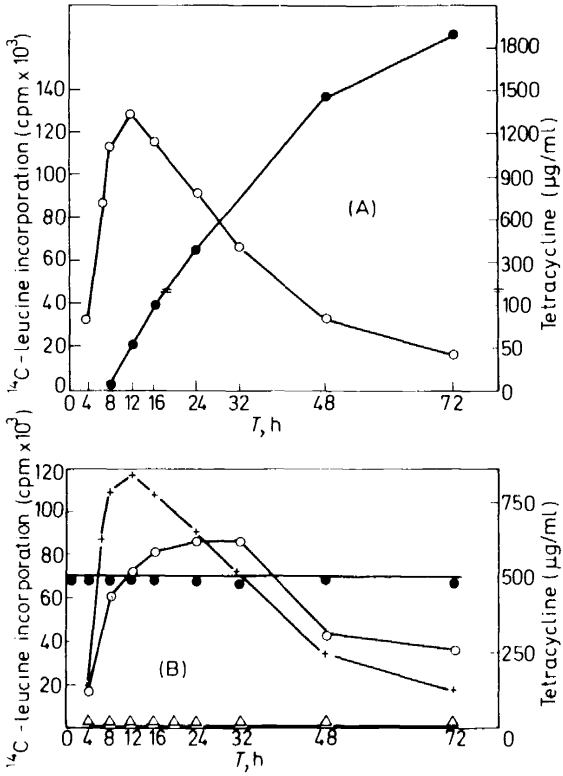


Figure 9. (A) Incorporation of ^{14}C-leucine into proteins of tetracycline-producing strain of *Streptomyces aureofaciens* (○—○) and production of tetracycline (●—●). (B) Effect of tetracycline on incorporation of ^{14}C-leucine into proteins. Course of ^{14}C-leucine incorporation after addition of tetracycline to 4h cultures: 50 μg ml^{-1} (+—+); 500 μg ml^{-1} (○—○) and 1000 μg ml^{-1} (△—△). In the experiment with 500 μg TC ml^{-1} the level of tetracycline was followed during the experiment (●—●)

inhibition of incorporation of ^{14}C-leucine into proteins and a complete inhibition of tetracycline synthesis[78]. Under the same experimental conditions, the TC-nonproducing strain had a completely blocked incorporation of leucine into proteins (*Figure 10*). It was found further that the nonproducing strain of *S. aureofaciens* is able to take up some 23 per cent more of ^{3}H-TC than the producing strain (*Figure 11*). The difference in the sensitivity of these strains to tetracycline can be partly accounted for by a difference in the permeability of the cells toward the antibiotic.

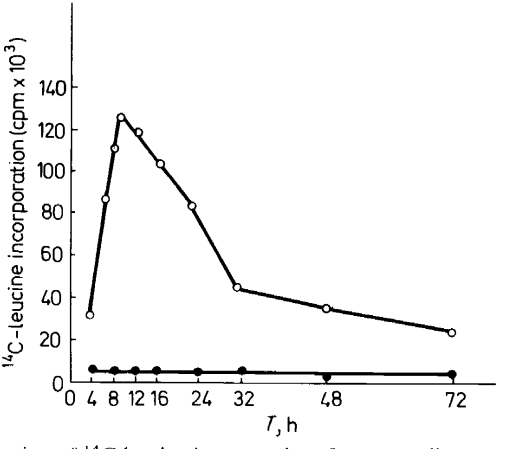


Figure 10. Incorporation of ^{14}C-leucine into proteins of a tetracycline-nonproducing strain of *Streptomyces aureofaciens*. ^{14}C-leucine incorporation in absence (○—○) and presence of 500 μg ml^{-1} tetracycline (●—●)

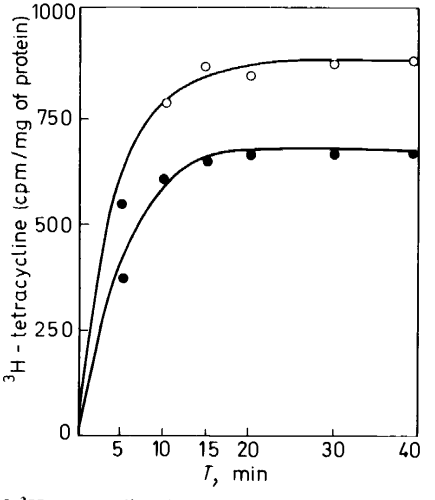


Figure 11. Uptake of ^{3}H-tetracycline by tetracycline-producing (●—●) and nonproducing (○—○) strains of *Streptomyces aureofaciens*. The 4h-old cells were incubated at 30°C with 500 μg ml^{-1} of ^{3}H-tetracycline (10 μCi). The samples were suspended in 10mM Tris–HCl buffer at pH 7.5 and filtered through a membrane filter. Radioactivity of washed cells was counted in a Bray's solution

Further results of studying protein synthesis in *S. aureofaciens* showed that addition of tetracycline during the exponential phase of growth leads to a partial inhibition of protein synthesis and a complete inhibition of tetracycline biosynthesis. Addition of tetracycline during the 16th hour of incubation had no effect on the production of the antibiotic. These results indicate that tetracycline interacts with the synthesis of specific enzymes which are essential for the synthesis of the antibiotic rather than affecting merely their activity[79]. One may conclude that the basis of the mechanism of action of these compounds and their interference with metabolism during the early hours of cultivation lies in an effect on protein synthesis or rather on the enzyme equipment of the cell.

It being established that under our cultivation conditions chlortetracycline is already formed after 10 hours of cultivation, we wanted to verify to what extent the activity of the enzyme systems of the so-called primary metabolism

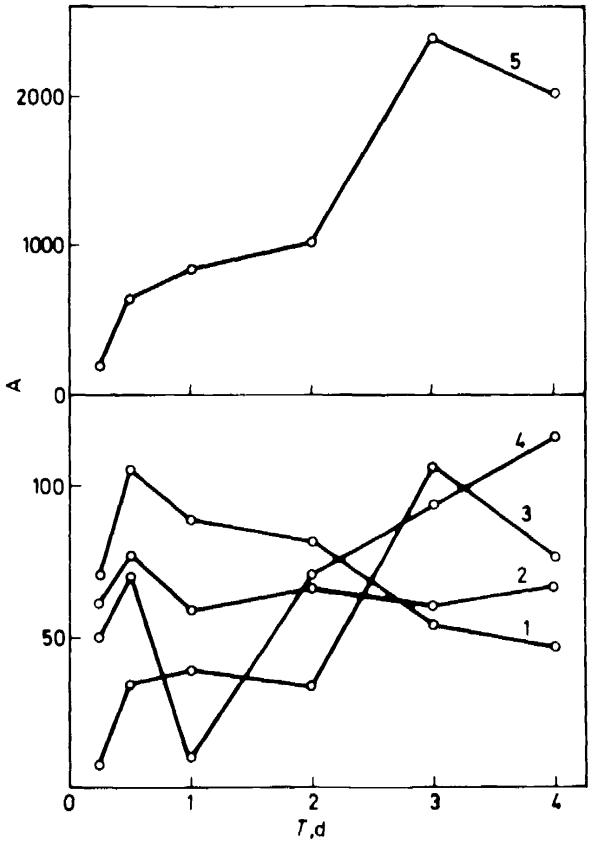


Figure 12. Changes in specific activities of tricarboxylic acid enzymes (A, μmol min^{-1} per mg protein) in the course of submerged cultivation of *S. aureofaciens* RIA 57 1—aconitate hydratase; 2—isocitrate dehydrogenase; 3—citrate synthase; 4—fumarate hydratase; 5—malate dehydrogenase

is affected. The dynamic growth of a *S. aureofaciens* culture on a reciprocal shaker sets in after some 6 hours and ceases after some 16 hours of cultivation. At this time, some of the Krebs cycle enzymes showed their maximum activity (*Figure 12*). Before estimating the enzyme activity we added 1 mg chlortetracycline ml^{-1} medium at this hour. It was observed that the activities of malate dehydrogenase and citrate synthase were not affected by the chlortetracycline added. The activities of ATPase, phosphoenolpyruvate carboxylase, malate dehydrogenase (decarboxylating) and isocitrate dehydrogenase were actually stimulated. On the other hand, acetyl-CoA carboxylase and pyruvate dehydrogenase were inhibited.

When adding chlortetracycline (for the actual estimation of enzyme activities) directly to the reaction mixture (50–200 $\mu g\ ml^{-1}$ reaction mixture) a stimulation of citrate synthase was observed, together with a slight inhibition of malate dehydrogenase and decarboxylating malate dehydrogenase, the other enzymes studied being without substantial changes. It was stated intentionally that the changes 'were observed' since there are many practical difficulties in preparing 'pure' enzyme preparations from *S. aureofaciens* that would not contain chlortetracycline which is present normally as a natural product of *S. aureofaciens* both in the cultivation medium and in the mycelium.

The inhibition of pyruvate dehydrogenase with chlortetracycline observed *in vitro* need not have an effect on the biosynthesis of this antibiotic. It is known that acetyl-CoA is formed either through oxidation of pyruvic acid or of fatty acids, inside the mitochondria. The question of existence of mitochondria in *Streptomycetes* is not unequivocally solved but still some observations indicate the existence of mitochondria-like forms in the cells. However, since most oligoketide-type compounds are probably synthesized by extra-mitochondrial agencies the transport of the two-carbon building unit across the mitochondrial membrane might play a role in the control of the synthesis of these compounds. The significance of the above acetyl-CoA deacetylase would then consist in its splitting acetyl-CoA to acetate which can be transported out of the mitochondria. However, other transport mechanisms have been envisaged, such as condensation with oxaloacetate and passage in the form of citrate, or condensation with carnitine and passage in the form of acetyl carnitine.

In conclusion to this section one might say that, in spite of the emphasis on the non-essential character of secondary metabolites (i.e. they do not form a common component of the cell protoplasm), it is admitted that their presence (either through synthesis or addition) during microbial culture growth can, even in minute amounts, (a) affect the structure of nucleic acids or replication, (b) inhibit the function of ribosomes, (c) suppress energy-transmitting reactions, and (d) damage membrane structure and activity[80]. This statement is admittedly very general and only further detailed work may help in elucidating to what extent the production of the antibiotic will participate in the regulation of the metabolic pathways of the producer itself.

EXCESSIVE METABOLITES

Let us briefly reiterate that the developmental cycle of a bacterial culture may be divided into several different phases. For example, lag, phase I, when the growth rate is equal to zero; acceleration, phase II, including the time when the cells begin to divide and when the rate of multiplication increases; exponential, phase III, when the growth rate is constant; retardation, phase IV; stationary, phase V, when the growth rate is again equal to zero. During phase V the bacteria multiply further but this is compensated by accelerated death rate. The next phase, of accelerated death rate, VI, represents a transition to the phase of exponential decrease, VII, which then terminates in the final phase VIII, which may be described by a straight line.

It is understandable that the curve of the various growth phases will be different for different bacterial species and will depend on the environmental conditions. If one discusses the formation of various microbial metabolites from the point of view of different growth phases it must not be forgotten that they are products, not only of bacteria, but also of *Streptomycetes*, fungi, algae, amoebas and other protozoa and these may differ very widely in their growth phases. In all attempts at generalization, one must keep in mind the difficulties arising from this complexity.

In a rough approximation, one may distinguish two metabolic phases, the anabolic and catabolic. Intensive synthesis of cell components during the anabolic phase passes over to the catabolic phase where decomposition and degradation processes begin to predominate. In microorganisms, this phase is further characterized by various synthetic processes which result, for example, in the formation of spores or of various polymers, pigments, and excessive formation of simple organic acids.

It is quite logical if one intercalates between the two phases, a third which is called the 'transition phase'. During this phase, the enzyme systems undergo a profound rearrangement, first of all from the point of view of their mutual regulation. Here one may speak of the trophophase, transition phase and idiophase[81], designating the metabolites formed during the trophophase as primary and those in the idiophase as secondary.

The terms primary and secondary metabolites are used mainly by plant physiologists. They distinguish between substances which can be detected in practically all plants and which possess obviously functional characters, such as chlorophyll or lipids, and substances which can be obtained only from certain plant species and which do not possess a clear general function, such as camphor and tannins.

The definition of secondary metabolites in microorganisms has further shortcomings. A secondary metabolite may be a substance with limited taxonomic distribution which is not formed under all conditions and which does not possess a clear metabolic function. However, among secondary

metabolites one may also count metabolites of primary metabolism (e.g. citric acid, vitamins, amino acids, nucleotides), if they are produced in disproportionate amounts[80].

This last view is somewhat embarrassing. One should remember that the existing genetic and physiological breeding methods of microbiology make it possible for virtually any metabolite at any phase of growth to be synthesized to excess. From the point of view of functional significance, it may be either essential or not. When studying the mechanisms controlling the biosynthesis of such a metabolite, its chemical structure is of decisive importance, together with the principal biosynthetic building blocks, the efficiency of enzyme systems which synthesize the metabolite, and its biological activity with all the regulating aspects which, under the given cultivation conditions, maintain the metabolite at a certain quantitative level. Experimental results indicate that the production of a metabolite in excess of normal is determined in a fundamental way not only by the genetic equipment of the microorganism but also by the environmental conditions during the so-called preparatory phase (lag phase). It is understandable that the conditions for the development of certain enzyme systems during further metabolic phases of microbial evolution will also be reflected either positively or negatively in the production ability.

If it is admitted that metabolic differentiation of microorganisms includes not only primary and secondary metabolism but that it also shows a deeper differentiation, then from the point of view of the control of biosynthesis of metabolites their division into primary and secondary metabolites has but a limited significance.

The aim of microbial syntheses is to maximize the formation of a certain product and to minimize the formation of other products[82]. In order to achieve excess production of a certain substance one must interfere with the normal control of metabolic pathways[83]. This can be achieved either by a gene mutation or by extreme conditions of the cultivation medium or by a combination of the two[84]. A planned interference with microbial syntheses depends on our knowledge of the possible ways of influencing the physiological phases of culture development.

Work dealing with the mechanisms controlling the biosynthesis of industrially important metabolites, work on the possibilities of circumventing these mechanisms, is of necessity at a pioneering stage. In no case can one agree with the view that during the transition phase a metabolic catastrophe must take place. This would be unphysiological and contrary to historical experience. Similarly, the designation of the production phase as an idiophase (particular phase), and of 'secondary' metabolites as biochemically bizarre molecules, does not contribute much to our understanding of the control mechanisms or of new metabolic equilibria which result in their overproduction.

Examples of mechanisms controlling the biosynthesis of industrially important metabolites as well as pathways for circumventing them have been reviewed several times[85,86]. In our attempts at quantifying a certain metabolite we are often enslaved by mechanistic views which follow from our knowledge of precursors and of individual biochemical steps leading to the synthesis of the desired compound. Regulation of biosynthesis as a

quantitative character is much more complex. Interference (genetic and physiological) with areas outside the main metabolic pathway may affect substantially the amount of substance produced[87,88].

It was the aim of this review to remove some of the mysteriousness from the mechanisms controlling the synthesis of secondary metabolites; to show that new information and data that have accumulated around the biosynthesis of these compounds will not fit under the heading 'secondary'. Above all, we wanted to stress the fact that the production of a given substance in quantitative terms is governed both by the genome of the microorganism and by the physiological conditions of the metabolic phases that have preceded the production phase.

METABOLIC AND EVOLUTIONARY CYCLE OF *S. aureofaciens*

It is generally assumed that the excessive (secondary) metabolites are not produced during so-called balanced growth. It has been claimed in a number of cases that balanced growth is terminated as nutrients like nitrogen or phosphorus are exhausted from the medium. The cases in point are the production of gibberellin by *Gibberella fujikuroi*[89], production of alkaloids by *Claviceps purpurea*[90], formation of anthraquinones by *Penicillium islandicum*[91] and others. A detailed study of the metabolism of microorganisms producing these excessive metabolites would certainly reveal that even here the statement is not fully justified.

The biosynthesis of chlortetracycline by *S. aureofaciens* proceeds differently. Inorganic phosphate is exhausted from the medium by the 10th hour of fermentation[33]. At that time also the maximum synthesis of RNA will take place[92]. The exhaustion of phosphate from the cultivation medium is a prerequisite for chlortetracycline biosynthesis. In no case, however, does it mean cessation of growth. On the contrary, after about 10 hours of cultivation, a vigorous multiplication of cells and an exponential growth of the culture take place[93,94]. In this case, one cannot speak of a termination of balanced growth (trophophase) due to exhaustion of nutrients since during cultivation of *S. aureofaciens* in cultivation flasks, sucrose and ammonia nitrogen become exhausted only after some 96 hours of cultivation. Under production conditions, nutrients are added to the medium to achieve higher yields of chlortetracycline. In spite of this, one may state on the basis of various enzyme assays that in the course of cultivation of *S. aureofaciens* profound enzyme changes take place. The beginning of rearrangement of enzyme systems (the transition phase) may then be placed at about 30 hours of cultivation. Minute qualitative and quantitative changes of the inner and outer medium, feedback control, induction and catabolite repression, will be reflected during subsequent cultivation in a vigorous synthesis of pigments, the chlortetracycline in which we are particularly interested being only one of the substances the production of which was pronouncedly altered.

Biosynthesis and excessive production of some metabolites does not close the metabolic and evolutionary cycle of the microorganism. The excessive metabolites can later be transformed to other compounds, or even degraded all the way to CO_2. New synthetic processes may be induced which lead to

the formation of new resistant forms, say, of spores, or else the cells and the whole culture will autolyze.

An idealized pattern of the metabolic phase of *S. aureofaciens* may be represented as follows†:

(i) *Preparatory* (lag) *phase* begins with the inoculation of fresh nutrient medium and ends after about 6 hours of cultivation. The external medium is of paramount importance for the metabolic phenotype of the young mycelium. It is known that a number of stimulators of the biosynthesis of tetracycline antibiotics will be effective only if added at the beginning of cultivation (at time zero). If these substances are added later during cultivation, say, after 24 hours, they have no effect on the biosynthesis of the antibiotic. The metabolic activity of the culture is rather low during this phase.

(ii) *Logarithmic phase* is characterized by a sharp rise of metabolic activity. It occurs between the 6th and the 16th hour of cultivation. During this phase the culture is most sensitive to changes of the outside medium (e.g., interruption of aeration between the 6th and the 12th hour). A number of enzyme systems (e.g. the tricarboxylic acid cycle) reach the maximum of their activity during the period. Similarly, the level of pyruvic acid in the culture reaches high values. The respiration of the high-production strains is pronouncedly higher than with the low-production strains. The amount of chlortetracycline varies about 200 $\mu g\, ml^{-1}$ cultivation medium. This phase is further characterized by exhaustion of inorganic phosphate. Nucleic acids are synthesized, the synthesis of RNA reaching here a maximum. Incorporation of ^{14}C-leucine into proteins rises up to the 12th hour of cultivation. An analogous pattern may be observed for the incorporation of ^{14}C-thymine into DNA. This phase is characterized also by a sharp rise of ATP concentration. The ATP level in cells reaches the first maximum during this phase, both in the nonproducing and in the high-producing strains.

The mycelium in the submerged culture is usually composed of strong, long, unbranched, basophilic hyphae.

(iii) *Stationary phase* cannot be sharply differentiated from the preceding phase but it is characterized by a drop of some enzyme activities, e.g. of aminoacyl-tRNA synthetase, the incorporation of ^{14}C-leucine into proteins, the decrease in activity of some tricarboxylic acid cycle enzymes, and the drop in the level of ATP. This phase is characterized by a rise of ATPase activity. Also the biosynthesis of fatty acids reaches a maximum here.

The culture contains fewer basophilic hyphae; after 24 hours, thin, very long, medium-basophilic hyphae predominate. In the course of further cultivation, a gradual decrease of the basophilic character of hyphae may be observed. The utilization of nitrogen and carbon sources by the culture proceeds evenly. This phase ends after about 30 hours of cultivation.

(iv) *Transition phase* is characterized by cessation of protein synthesis. At a concentration of about 500 μg CTC ml^{-1} culture, the ribosomes become

† The time values shown serve merely for orientation. The course of cultivation and of enzyme activity and production maxima will differ, depending on metabolic and cultivation conditions and on the microbial strain used. Transitions between phases in a heterogeneous culture are not as sharp and the individual phases may overlap.

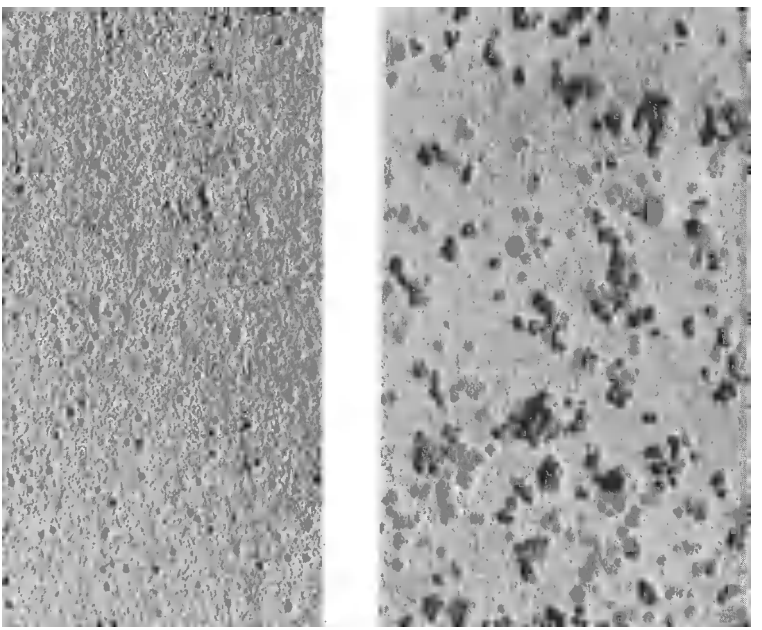


Figure 13. Isolated ribosomes from the cells of *S. aureofaciens*, producing strain 84/25 from 8h cultures (left). Ribosomal aggregates isolated from the same TC producing strain *S. aureofaciens* after 48 hours of cultivation (right). Magnification 40000 ×

Figure 14. Surface part of submerged mycelium of *S. aureofaciens*, 36 hours old, observed by scanning electromicroscope Geen 50A. Shadowed with gold, enlargement 10000 × in original, reduced by one-half

aggregated (*Figure 13*). It appears that during this period, feedback and induction mechanisms become dominant which control the synthesis of chlortetracycline. After about 40 hours, metachromatic granules of volution begin to appear in the hyphae and their number rises considerably up to the 72nd hour. During this phase, the ATPase activity reaches its maximum. The culture contains no more basophilic hyphae and only long thin filaments persist (*Figure 14*). This phase may be considered to end after about 45 hours of cultivation.

(v) *Excess phase.* During this phase, which may be placed somewhere between the 45th and the 96th–120th hour of cultivation, a vigorous biosynthesis of the excess metabolite may take place. The metabolic activity of the culture during this phase is heavily dependent on the genotype. With low-production strains resembling the standard type, this phase is accompanied by a renewed increase of activity of the tricarboxylic acid cycle, concomitant with a rising level of ATP in cells. The source of carbon in the medium is still intensively utilized but it is mostly oxidized in the tricarboxylic acid cycle and the ATP is apparently transformed to polyphosphate.

The metabolic activity of the producing strains is concentrated during this phase on the biosynthesis of the antibiotic. In contrast with the low-production strains, the activity of the TCA cycle and the ATP level do not rise substantially. During this phase, the production strain forms a characteristic mycelium, composed of very thin, branched hyphae which contain no metachromatic granules. It is likely that due to genetic changes in the energy metabolism, the high-production strain displays a decreased activity of the TCA cycle and the precursors thus spared are utilized as building blocks for the biosynthesis of tetracyclines.

(vi) *Degradation phase, autolysis.* During this phase the biosynthetic activities cease. Accumulation of the excess metabolite induces enzyme systems which result in its degradation. Frequently, these processes are accompanied by an autolysis of the mycelium. The metabolic activity of the culture stops, the content of ATP in the mycelium drops sharply, the mycelial hyphae are fragmented into small pieces. Later, the hyphae lyse completely.

CONCLUSION

The experimental data permit the conclusion to be drawn that chlortetracycline does not fit into the category of the so-called 'secondary metabolites'. The study of regulation processes controlling the biosynthesis of chlortetracycline stresses the importance of the metabolic phases which precede the actual production phase. The view is advanced that the existing physiological and genetic approaches to microbiology make it possible for cell metabolites, such as vitamins, amino acids, organic acids, antibiotics and others, to become 'excess metabolites'. To attain the excess character of production of a given metabolite is the aim of studying metabolic pathways and control mechanisms. For this reason we feel that the term excess metabolite expresses better the present state of information in industrial microbiology than does the term 'secondary metabolite'.

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REFERENCES

[1] A. J. Birch, *Fortschr. Chem. Org. Naturst.* **14**, 186 (1957).
[2] A. J. Birch, *Science* **156**, 202 (1967).
[3] J. D. Bu'Lock, *The Biosynthesis of Natural Products*. McGraw-Hill, London (1965).
[4] J. H. Rickards and J. B. Hendrickson, *The Biosynthesis of Steroids, Terpenes and Acetogenins*. W. A. Benjamin, New York (1964).
[5] Z. Vaněk and I. Málek, In *Global Impacts of Applied Microbiology* (M. P. Starr, ed.). p. 382, Almquist and Wiksell, Stockholm (1964).
[6] K. Aghoramurthy and T. R. Seshadri, *J. Sci. Ind. Res. (India)* **13A**, 114 (1954).
[7] H. Raistrick, *Suomen Kemistilehti* **23**, 221 (1950); *Acta Chem. Fennica* **10A**, 221 (1950).
[8] E. L. Tatum, *Ann. Rev. Biochem.* **13**, 667 (1944).
[9] G. Ehrensvärd and S. Gatenbeck, *Intern. Kongr. reine u. Angew. Chem.* **2**, 99. Verlag Chemie, Weinheim (1960).
[10] S. Gatenbeck, *Acta Chem. Scand.* **11**, 555 (1957).
[11] R. Thomas, *Biochem. J.* **78**, 748 (1961).
[12] F. Lynen and M. Tada, *Angew. Chem.* **73**, 513 (1961).
[13] Z. Vaněk and M. Souček, *Fol. microbiol.* **7**, 262 (1962).
[14] P. Dimroth, H. Walter and F. Lynen, *Europ. J. Biochem.* **13**, 98 (1970).
[15] E. W. Bassett and S. W. Tanenbaum, *Biochim. Biophys. Acta* **40**, 535 (1960).
[16] S. W. Tanenbaum and E. W. Bassett, *Biochim. Biophys. Acta* **59**, 524 (1962).
[17] S. Gatenbeck and S. Hermodsson, *Acta Chem. Scand.* **19**, 65 (1965).
[18] S. Gatenbeck, *Biochem. Biophys. Res. Comm.* **6**, 422 (1961).
[19] J. R. D. McCormick, In *Biogenesis of Antibiotic Substances* (Z. Vaněk and Z. Hošťálek, eds.) p. 73. Publ. House of the Czech. Acad. Sci., Prague and Academic Press, New York (1955).
[20] J. R. D. McCormick, In *Antibiotics, vol. II* (D. Gottlieb and P. D. Shaw, eds.) p. 113, Springer Verlag, Berlin, Heidelberg and New York (1967).
[21] L. A. Mitscher, *J. Pharmaceut. Sci.* **57**, 1633 (1968).
[22] J. F. Snell, A. J. Birch and P. L. Thomson, *J. Am. Chem. Soc.* **82**, 2402 (1960).
[23] J. F. Snell, R. L. Wagner and F. A. Hochstein, *Proc. Intern. Conf. Peaceful Uses Atom. Energy, Geneva*, vol. 12. Columbia Univ. Press (I.D.S.) New York (1955).
[24] P. A. Miller, J. R. D. McCormick and A. P. Doerschuk, *Science* **123**, 1030 (1956).
[25] H. Turley and J. F. Snell, *Biosynthesis of Tetracycline Antibiotics*. (J. F. Snell, ed.) vol. 1, p. 95. Academic Press, New York and London (1966).
[26] E. R. Catlin, C. H. Hassall and D. R. Parry, *J. Chem. Soc. C* 1363 (1969).
[27] A. J. Birch, C. J. Moye, R. W. Rickards and Z. Vaněk, *J. Chem. Soc.* 3586 (1962).
[28] A. T. Hudson, I. M. Campbell and R. Bentley, *Biochemistry* **9**, 3988 (1970).
[29] J. R. Brown, M. S. Spring and J. R. Stoker, *Phytochemistry* **10**, 2059 (1971).
[30] S. A. Waksman, *The Actinomycetes*. Vol. I, p. 157. The Williams and Wilkins Co. Baltimore (1959).
[31] E. Masno and E. Kondo, *Nippon Nogie-kagaku Kaishi* **29**, 555 (1955); Chem. Abstr. **51**, 8879 (1957).
[32] H. B. Woodruff and J. W. Foster, *J. Bacteriol.* **45**, 30 (1943).
[33] J. Doskočil, Z. Hošťálek, J. Kašparová, J. Zajíček and M. Herold, *J. Biochem. Microbiol. Technol. Eng.* **1**, 261 (1959).
[34] J. Voříšek, A. J. Powell and Z. Vaněk, *Fol. microbiol.* **14**, 398 (1969).
[35] J. Voříšek, A. J. Powell and Z. Vaněk, *Fol. microbiol.* **15**, 153 (1970).
[36] Z. Hošťálek, I. A. Ryabushko, J. Cudlín and Z. Vaněk, *Fol. microbiol.* **14**, 121 (1969).
[37] Z. Hošťálek, V. Jechová, M. Blumauerová and Z. Vaněk, *Biotechnol. Bioeng.* **11**, 539 (1969).
[38] F. A. Hochstein, *J. Org. Chem.* **24**, 679 (1959).
[39] V. Jechová, Z. Hošťálek and Z. Vaněk, *Fol. microbiol.* **14**, 128 (1969).
[40] E. J. Bormann and R. Herrmann, *Arch. Mikrobiol.* **63**, 41 (1968).
[41] H. Goldfine and E. R. Standtman, *J. Biol. Chem.* **235**, 2238 (1960).
[42] O. Hayaishi and A. Kornberg, *J. Biol. Chem.* **197**, 717 (1952).

[43] H. E. Pattee, L. M. Shannon and J. Y. Lew, *Nature* **201**, 1328 (1964).
[44] L. M. Shannon, J. de Vellis and J. Y. Lew, *Plant Physiol.* **38**, 691 (1963).
[45] J. de Vellis, L. M. Shannon and J. Y. Lew, *Plant Physiol.* **38**, 686 (1963).
[46] W. C. Hülsmann, *Biochim. Biophys. Acta* **77**, 502 (1963).
[47] W. C. Hülsman, *Biochim. Biophys. Acta* **125**, 397 (1966).
[48] R. W. Swick and H. G. Wood, *Proc. Nat. Acad. Sci. USA.* **46**, 28 (1960).
[49] *Základy biochemie*, p. 184. NČSAV Academia (In Czech) Prague (1965).
[50] J. D. Brodie, G. Wasson and J. W. Porter, *J. Biol. Chem.* **239**, 1346 (1963).
[51] D. J. H. Brock and K. Bloch, *Biochem. Biophys. Res. Commun.* **23**, 775 (1966).
[52] T. M. Harris, C. M. Harris and R. J. Light, *Biochim. Biophys. Acta* **121**, 420 (1966).
[53] R. J. Light, T. M. Harris and C. M. Harris, *Biochemistry* **5**, 4037 (1966).
[54] T. E. Acker, P. E. Brenneisen and S. W. Tanenbaum, *J. Am. Chem. Soc.* **88**, 834 (1966).
[55] R. Bentley and P. M. Zwitkowits, *J. Am. Chem. Soc.* **89**, 676 (1967).
[56] R. Bentley and P. M. Zwitkowits, *J. Am. Chem. Soc.* **89**, 681 (1967).
[57] P. E. Brenneisen, T. E. Acker and S. W. Tanenbaum, *J. Am. Chem. Soc.* **86**, 1264 (1964).
[58] S. W. Tanenbaum, S. Nakajima and G. Marx, *Biotechnol. Bioeng.* **11**, 1135 (1969).
[59] F. A. Hochstein, S. M. von Wittenau, F. W. Tanner, Jr. and K. Murai, *J. Am. Chem. Soc.* **82**, 5934 (1960).
[60] V. Běhal, J. Cudlín and Z. Vaněk, *Fol. microbiol.* **14**, 117 (1969).
[61] V. Běhal and J. Jílek, *Fol. microbiol.* **14**, 211 (1969).
[62] V. Běhal, V. Procházková and Z. Vaněk, *Fol. microbiol.* **14**, 112 (1969).
[63] M. Podojil, Z. Vaněk, B. Běhal and M. Blumauerová, Regulation of biosynthesis of secondary metabolites. XIV. *Fol. microbiol.* (to be published).
[64] K. Mikulík *et al.*, *Fol. microbiol.* **14**, 232 (1969).
[65] S. I. Alikhanyian, *Selekcia promyshlennykh mikroorganizmov.* (In Russian). Publ. House Nauka, Moscow (1968).
[66] N. S. Egorov, E. G. Toropova, A. A. Biltrikova and O. A. Egorova, *Antibiotiki* **16**, 675 (1971).
[67] G. Sermonti, *Genetics of Antibiotic-Producing Microorganisms.* Wiley-Interscience (1969).
[68] K. Katagiri, *J. Antib.* **A7**, 45 (1954).
[69] S. I. Veselova, *Genetika* **3**, 73 (1967).
[70] S. I. Veselova and L. V. Komarova, *Genetika* **4**, 100 (1968).
[71] S. A. Waksman, H. C. Reilly and D. Johnstone, *J. Bacteriol.* **52**, 393 (1946).
[72] M. Legator and D. Gottlieb, *Antib. Chemother.* **3**, 809 (1953).
[73] D. A. Lowe and D. W. S. Westlake, *Canad. J. Biochem.* **49**, 448 (1971).
[74] V. S. Malik and L. C. Vining, *Canad. J. Microbiol.* **16**, 173 (1970).
[75] G. Boretti and F. Raggi, *Giorn. microbiol.* **1**, 224 (1955).
[76] Z. Hošťálek, *Fol. microbiol.* **9**, 78 (1964).
[77] Z. Hošťálek, J. Janeček, J. Doskočil and J. Kašparová, *Naturwissenschaften* **45**, 1 (1958).
[78] K. Mikulík, J. Karnetová, A. Křemen, J. Tax and Z. Vaněk, *Radiation and Radioisotopes for Industrial Microorganisms*, p. 201. International Atomic Energy Agency, Vienna (1971).
[79] K. Mikulík, J. Karnetová, N. Quyen, M. Blumauerová, I. Komersová and Z. Vaněk, *J. Antib.* **24**, 801 (1971).
[80] E. D. Weinberg, *Perspectives in Biology and Medicine* **14**, 565 (1971).
[81] J. D. Bu'Lock and A. J. Powell, *Experientia* **21**, 55 (1965).
[82] S. J. Pirt, *Symposia of the Society for General Microbiology No. XIX. Microbial Growth*, p. 199 (1969).
[83] J. Nüesch, *Path. Microbiol.* **34**, 158 (1969).
[84] R. Hütter, *Path. Microbiol.* **34**, 195 (1969).
[85] A. L. Demain, *Lloydia* **31**, 395 (1968).
[86] I. Málek, In, *About Mechanisms Regulating the Biosynthesis of Secondary Metabolites in Biogenesis of Antibiotic Substances*, (Z. Vaněk, Z. Hošťálek, eds.), p. 11. Publ. House of the Czech. Acad. Sci. Prague (1965).
[87] Z. Hošťálek and Z. Vaněk, In, *Genetics of Industrial Microorganisisms, Actinomycetes and Fungi.* (Z. Vaněk, Z. Hošťálek and J. Cudlín, eds.), p. xxx. Academia—Elsevier (1972).
[88] Z. Vaněk, J. Cudlín, M. Blumauerová and Z. Hošťálek, *Fol. microbiol.* **16**, 225 (1971).
[89] A. Borrow, E. G. Jeffery, R. H. J. Kessel, E. C. Lloyd, P. B. Lloyd and I. S. Nixon, *Canad. J. Microbiol.* **7**, 227 (1961).
[90] L. C. Vining and W. A. Taber, In, *Biochemistry of Industrial Microorganisms.* (C. Rainbow and A. H. Rose, eds.) p. 341. London (1963).

[91] S. Gatenbeck, *Studies on the Basic Metabolism Determining the Biosynthesis of Malonate Derived Compounds in P. Islandicum in Biogenesis of Antibiotic Substances.* Publ. House Czech. Acad. Sci., Prague (1965).
[92] M. A. Guberniev, N. A. Ugoleva and L. I. Torbochkina, *Antibiotiki* **1**, 8 (1956).
[93] G. Biffi, G. Boretti, A. DiMarco and P. Pennella, *Appl. Microbiol.* **2**, 288 (1954).
[94] G. Boretti, A. DiMarco, T. Scotti and P. Zocchi, *Giorn. microbiol.* **1**, 97 (1955).