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Metabolic Regulation in Tylosin-Producing *Streptomyces fradiae*: Regulatory Role of Adenylate Nucleotide Pool and Enzymes Involved in Biosynthesis of Tylonolide Precursors

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The relationship was studied between the level of the intracellular adenylates and the biosynthesis of tylosin by *Streptomyces fradiae* NRRL 2702. The adenylate level was observed to be inversely related to the rate of tylosin biosynthesis and hence the final concentration of the antibiotic. The concentrations of the adenylates were maximal during the trophophase, dropped quickly before the onset of tylosin biosynthesis, and remained at low levels throughout the idiophase. The adenylate energy charge was almost constant throughout the fermentation and was in the range of 0.4 to 0.55. Glucose addition in the idiophase suppressed tylosin biosynthesis, accompanied by a rapid increase in the adenylate levels. The biosynthesis of tylosin resumed after a rapid drop in the adenosine triphosphate concentration. Two enzymes catalyzing the interconversion of propionyl-coenzyme A and methylmalonyl-coenzyme A were found in this organism: methylmalonyl-coenzyme A carboxyltransferase (EC 2.1.3.1) and propionyl-coenzyme A carboxylase (EC 6.4.1.3). The activity of the former was two orders of magnitude higher than that of the latter. The activities of both enzymes were affected by the increased glucose addition in the idiophase.

Tylosin, a macrolide antibiotic synthesized by *Streptomyces fradiae*, was first described by McGuire et al. in 1961 (9). Later investigations showed that the organism coproduced four antibiotics with structures closely related to tylosin (6). Seno et al. (14) presented data showing that the major factors formed by *S. fradiae* were tylosin, macrocin, and relomycin (Fig. 1). These compounds occur in the terminal stages of tylosin biosynthesis, and a pathway relating the compounds has been proposed (14).

In a previous study (P. P. Gray and S. Bhuwapathanapun, Biotechnol. Bioeng., in press), chemostat cultures were used to determine the relationship between the specific uptake rates of glucose, glycerol, phosphate, and sodium glutamate on the biosynthesis of tylosin and related compounds.

In this study, shake flask cultures have been used to investigate the relationship between tylosin biosynthesis and the intracellular concentrations of adenosine triphosphate, diphosphate, and monophosphate (ATP, ADP, and AMP) and the adenylate energy charge in normal batch culture and after the addition of glucose to idiophase cultures. The 16-membered lactone ring of tylosin has been shown (11) to be derived from two acetates, five propionates, and one butyrate. In light of the importance of propionyl-coenzyme A (CoA) as a precursor for lactone ring synthesis, the presence of enzymes interconverting methylmalonyl CoA and propionyl CoA was investigated. Activities of methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1) and propionyl CoA carboxylase (EC 6.4.1.3) were followed throughout a normal batch fermentation and after the metabolic stress caused by glucose addition during the idiophase.

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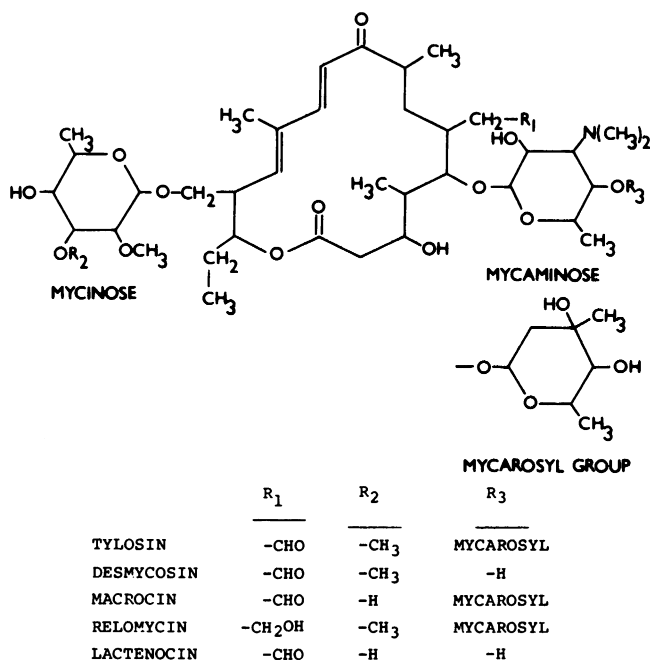
MATERIALS AND METHODS

Organism and cultivation. The experiments in this study were conducted in shake flasks by using *S. fradiae* NRRL 2702 in the modified Stark no. 8 medium (Gray and Bhuwapathanapun, in press) which contained in 1 liter: NaCl, 2.0 g; $MgSO_4$, 5.0 g; $CoCl_2 \cdot 5H_2O$, 0.001 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; ferric ammonium citrate, 3.0 g; Betaine hydrochloride, 5.0 g; L-sodium glutamate, 20.0 g; glucose, 35.0 g; $CaCO_3$, 3.0 g; methyloleate, 3.0 g; and K_2HPO_4, 2.3 g. Growth conditions and assay procedures were as previously described (Gray and Bhuwapathanapun, in press).

Determination of adenylates (ATP, ADP, and AMP). Adenylates were released from the fermentation culture by using the extraction method described by J. H. Baker (M.S. thesis, University of New South Wales, Kensington, New South Wales, Australia, 1977) and were assayed according to the luciferase method of Pradet (12), later modified by Chapman and coworkers (2).



	R_1	R_2	R_3
TYLOSIN	-CHO	$-CH_3$	MYCAROSYL
DESMYCOSIN	-CHO	$-CH_3$	-H
MACROCIN	-CHO	-H	MYCAROSYL
RELOMYCIN	$-CH_2OH$	$-CH_3$	MYCAROSYL
LACTENOCIN	-CHO	-H	-H

FIG. 1. *Chemical structures of the tylosin fermentation components.*

Extraction. A 1.0-ml sample of the culture was pipetted into a 50-ml Erlenmeyer flask containing 25 ml of vigorously boiling reagent solution. The reagent solution (pH 7.75) contained 20 mM tris(hydroxymethyl) aminomethane (Tris) and 2 mM ethylenediaminetetraacetate.

The culture sample was extracted for 90 s and cooled down to 0°C on ice. The extraction solution was then refilled to the final volume of 25 ml and kept frozen until assayed (Baker, M.S. thesis).

Assay procedure. ATP was determined by means of the luciferase reaction (18) with an integrating photometer (model 3000, serial 6042, SAI Technology).

Luciferase (Sigma Chemical Co.) was prepared by filling the vials containing lyophilized lantern extract with sterile distilled water and was incubated at 4°C for 24 h. The enzyme solution was centrifuged to remove debris before assay.

The reaction mixture for ATP determination contained 400 μl of cell extract and 100 μl of a 75 mM potassium phosphate buffer (pH 7.3) containing 15 mM $MgCl_2$.

For ATP plus ADP determinations, 400 μl of the cell extract was added to 100 μl of a solution containing 75 mM potassium phosphate (pH 7.3), 15 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate (Boehringer Mannheim Corp.) and 20 μg of pyruvate kinase (EC 2.7.1.40; Boehringer Mannheim).

For total adenylate determinations, 400 μl of the cell extract was added to 100 μl of a solution containing 75 mM potassium phosphate (pH 7.3), 15 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 20 μg of pyruvate kinase and 25 μg of adenylate kinase (EC 2.7.4.3; Boehringer Mannheim).

The three mixtures were incubated at 30°C for 15 min and then held at 0°C until assayed. The adenylate kinase was dialyzed against 50 mM potassium phosphate buffer (pH 7.3) for 24 h before use.

A volume of 500 μl of the incubated mixtures was mixed well with 500 μl of luciferase in a 20-ml sampling vial for about 15 s before being measured. The standard curve for ATP was constructed each time the method was used.

The concentrations of the adenylates were expressed as milligrams of adenylate per gram (dry weight) of the respective sample. Adenylate energy charge was calculated by the method of Atkinson and Walton (1).

Assay of propionyl-CoA carboxylase (EC 6.4.1.3) and methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1) activities. The activity of propionyl-CoA carboxylase was determined by the method of Tietz and Ochoa (19) and that of methylmalonyl-CoA carboxyltransferase was determined by the method of Wood and Stjernholm (22).

Preparation of cell extract. Contents of 1 or 2 fermentation flasks were pooled, and the mycelium was collected by centrifugation at 10,000 × *g* for 15 min at 4°C, washed several times with distilled water to remove methyloleate and then washed twice with 50 mM Tris buffer (pH 8.0). A 5- to 6-g amount (wet weight) of mycelium was suspended in 17 ml of 50 mM Tris buffer (pH 8.0) containing 10 mM 2-mercaptoethanol and held below 5°C in ice. The cell suspension was quickly transferred to a 50-ml Braun homogenization bottle containing 30 g of glass beads (0.17 to 0.18 mm in diameter), which were previously washed twice with 50 mM Tris buffer (pH 8.0).

The mycelium was then disrupted, using a B Braun

homogenizer (Melsunger AG, type 853034, West Germany). Disrupted cell suspensions were centrifuged at 16,000 × *g* for 15 min at 0°C. The supernatant obtained was used for enzyme assay and for protein estimation.

Assay of methylmalonyl-CoA carboxyltransferase. The activity of the enzyme was measured by determining pyruvate formed from oxaloacetate. The reaction mixture (13) contained, in 2 ml: Tris buffer (pH 8.0), 100 μmol; reduced glutathione (Sigma), 1 μmol; oxaloacetate (Calbiochem), 2 μmol; propionyl-CoA, prepared from propionic anhydride by the method of Simon and Shemin (16), 0.5 μmol; and cell-free extract containing 2 to 3 mg of protein. In the control, propionyl-CoA was omitted.

After 15 min of incubation at 30°C, the reaction was terminated by the addition of 1 ml of 10% $HClO_4$. The mixture was cooled in ice and centrifuged, and the neutralized supernatant was used to determine pyruvate with lactate dehydrogenase (2).

Assay of propionyl-CoA carboxylase. The enzyme activity was determined by measuring the acyl-CoA-dependent formation of acid-stable radioactivity derived from $NaH^{14}CO_3$. The incubation mixture (21) contained, in 2 ml: Tris buffer, pH 8.0, 100 μmol; ATP, 2.5 μmol; $MgCl_2$, 5 μmol; NaF, 10 μmol; $NaH^{14}CO_3$ (Amersham), 10 μCi (59.7 μCi/μmol); reduced glutathione, 1 μmol; propionyl-CoA, 0.5 μmol; and cell-free extract containing 2 to 3 mg of protein. The mixture was incubated for 30 min at 30°C, and the reaction was stopped by adding 1.0 ml of 2 N HCl. The precipitated protein was removed by centrifugation and 1.0 ml of the supernatant was dried and then used for counting. A 10-ml volume of scintillation solution was added to each sample, and the radioactivity was counted on a Packard Tri-Carb Liquid Scintillation Spectrometer (model 2650; Packard Instrument Co.). The control was run without propionyl-CoA.

Tylosin assay (total factors). Tylosin in the fermentation broth was determined spectrophotometrically after being extracted with chloroform by the method described by Caltrider and Hayes (P. G. Caltrider and H. B. Hayes, U.S. patent 3,433,711, 1969). A volume of 10 ml of culture was centrifuged at 1,300 × *g* for 15 min. A 5-ml amount of the supernatant broth which was previously filtered was adjusted to pH 5.0 with 0.1 N H_2SO_4, then diluted to 10 ml with acidified distilled water. A 2-ml volume of the acidified broth was stirred with 50 ml of chloroform for 5 min. Tylosin in the chloroform extract was determined spectrophotometrically at 283 nm.

Protein determination. Protein was estimated by the Folin-Ciocalteu method, using bovine serum albumin (Sigma) as standard.

RESULTS

The growth charactersitics of a tylosin-producing batch culture of *S. fradiae* are shown in Fig. 2. Mycelial growth reached the stationary phase after 5 days of cultivation, attaining a cell concentration of 30 g (dry weight) per liter. Glucose and sodium glutamate were rapidly utilized during the initial growth phase; they were practically undetectable after 3 days of fermentation. On the contrary, methyloleate was utilized during the idiophase.

The pH was decreased slightly during the early period of growth, then gradually increased, reaching about 9.0 by the end of the fermentation.

Very little tylosin was synthesized during the trophophase. The onset of tylosin synthesis occurred on day 4 to 5, and at the end of the fermentation the tylosin concentration was approximately 2.0 g/liter.

Level of the adenylates and the adenylate energy charge. Intracellular concentrations of ATP and ADP reached their maximal values during period of rapid growth. ATP and ADP remained at high levels up to 4 days of cultiva-

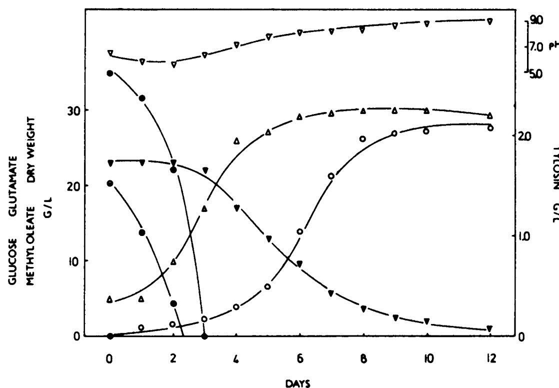


FIG. 2. *Batch growth characteristics of tylosin-producing S. fradiae. Symbols: ▽, pH; ○, glucose; ◒, sodium glutamate; ▼, methyloleate; △, cell dry weight; ○, tylosin.*

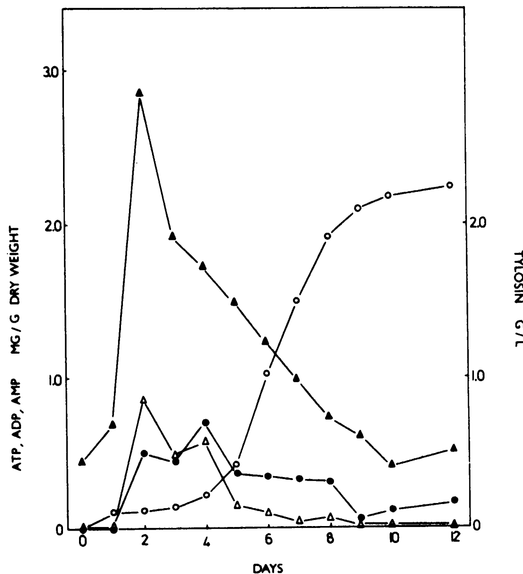


FIG. 3. *Intracellular concentrations of adenylates during the batch fermentation of tylosin. Symbols: △, ATP; ▲, ADP; ●, AMP; ○, tylosin.*

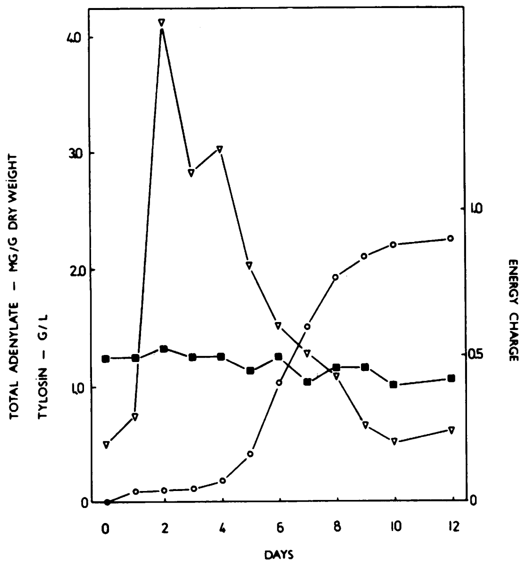


FIG. 4. *Energy charge and total intracellular adenylate during the batch fermentation of tylosin. Symbols:* ▽, *total adenylate;* ■, *energy charge;* ○, *tylosin.*

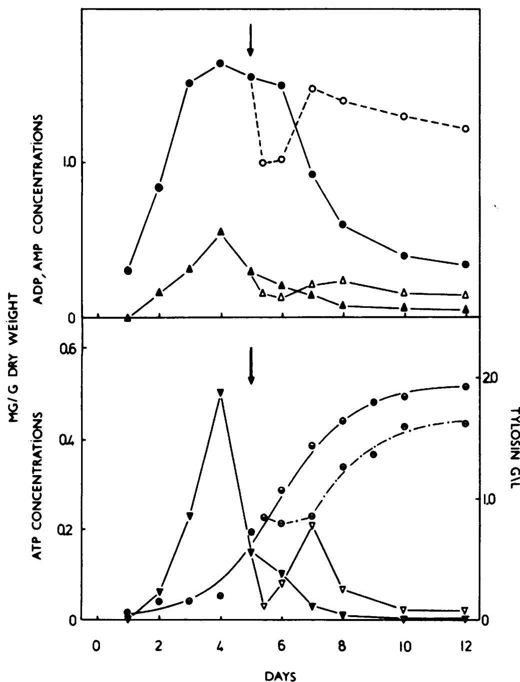


FIG. 5. *Effect of glucose addition (30 g/liter) in idiophase on the intracellular adenylates. Closed symbols, control flasks; open symbols, flasks with added glucose. Symbols:* ▼, ▽, *ATP;* ●, ○, *ADP;* ▲, △, *AMP;* ⊖, *tylosin, control;* ⊖, *tylosin with addition of glucose.*

tion and dropped rapidly before the onset of tylosin synthesis (Fig. 3). The maximum concentration of AMP occurred at 4 days. The concentrations of ATP and AMP were always significantly lower than that of ADP throughout the fermentation. The sum total of adenylates also displayed a similar pattern, characterized by a rapid decrease before the onset of antibiotic synthesis (Fig. 4).

The energy charge was almost constant throughout the fermentation and in the range of 0.44 to 0.55 (Fig. 4).

Effect of glucose on the adenylate level and energy charge. Tylosin biosynthesis in a batch fermentation commenced just before the exhaustion of glucose, and glucose remained undetectable in the idiophase. During this period, methyloleate was the major carbon source. The effect of addition of glucose in the idiophase was reported previously (Gray and Bhuwapathanapun, in press). The addition of 30 g of glucose per liter to 5-day tylosin-producing cultures caused a transient decrease, followed by a sharp increase in the concentration of ATP (Fig. 5).

The levels of ADP and AMP also displayed similar patterns and remained at increased values until the end of the fermentation. Tylosin biosynthesis resumed after a rapid drop in ATP concentration (Fig. 5). The energy charge was almost unchanged after the addition of glucose in idiophase (Fig. 6).

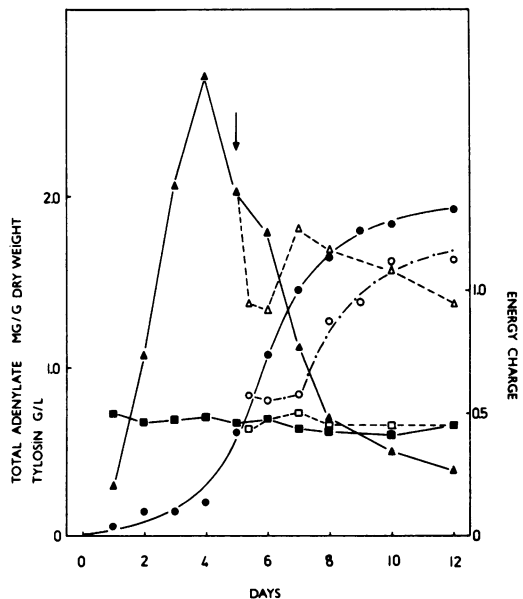


FIG. 6. *Effect of glucose addition (30 g/liter) in idiophase on the total adenylate and energy charge. Closed symbols, control flasks; open symbols, flasks with added glucose. Symbols:* ▲, △, *total adenylate;* ■, □, *energy charge;* ●, ○, *tylosin.*

Occurrence of the enzymes involved in the interconversion of propionyl-CoA and methylmalonyl-CoA. The activity of two carboxylating enzymes in tylosin-producing *S. fradiae* was observed: propionyl-CoA carboxylase (EC 6.4.1.3) and methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1). Both enzymes showed their highest activities after 5 to 6 days of fermentation (Fig. 7). The activity of the carboxyltransferase was higher than that of the carboxylase by about two orders of magnitude. The specificity of the two enzymes has not been determined.

Glucose was observed to exert a depressive effect on the carboxylase and carboxyltransferase (Fig. 8). The study of the inhibition/repression effect caused by increased glucose addition on the carboxyltransferase (data not shown) demonstrated that the enzyme probably suffered from repression rather than inhibition.

DISCUSSION

The possible involvement of ATP in controlling antibiotic production was suggested by the data of Silaeva et al. (15) and Janglova et al. (7)

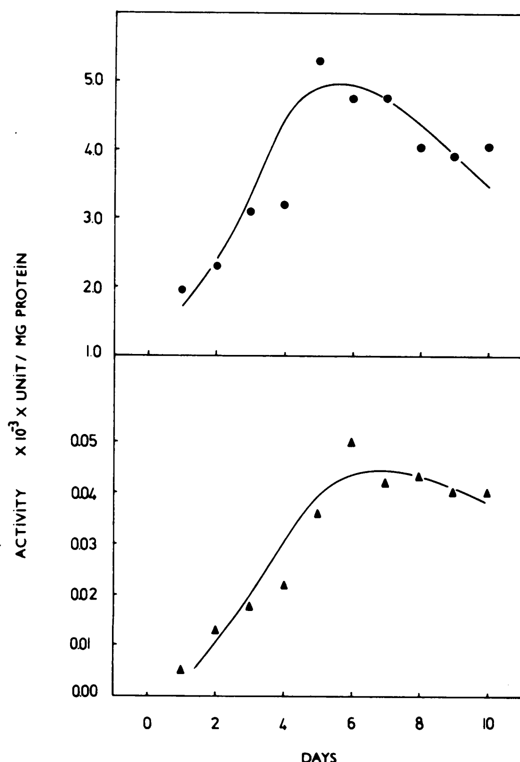


Fig. 7. *Activities of the enzymes catalyzing the interconversion of methylmalonyl-CoA and propionyl-CoA. Symbols: ●, methylmalonyl-CoA carboxyltransferase; ▲, propionyl-CoA carboxylase.*

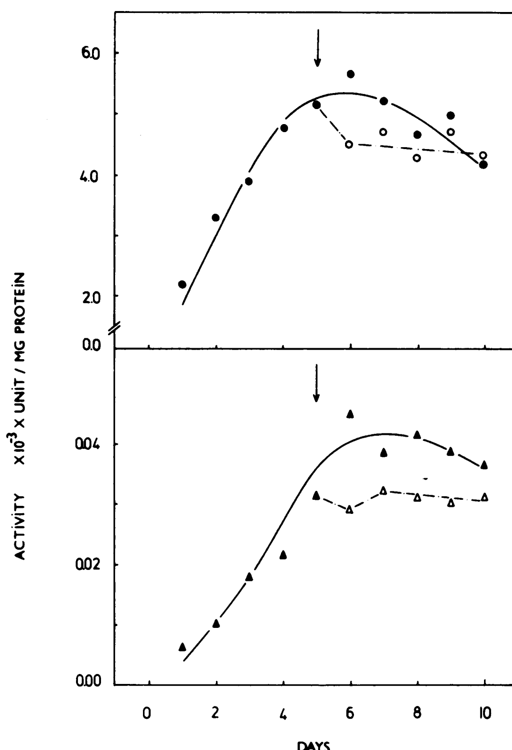


Fig. 8. *Effect of glucose addition (30 g/liter) in idiophase on the activities of methylmalonyl-CoA carboxyltransferase and propionyl-CoA carboxylase. Glucose was added on day 5. Closed symbols, control flasks; open symbols, flasks with added glucose. Symbols: ●, ○, methylmalonyl-CoA carboxyltransferase; ▲, △, propionyl-CoA carboxylase.*

in *Bacillus brevis* and *S. aureofaciens*, respectively. ATP was also suggested to be involved in the regulation of tyrothricin (5) and candicidin (8) biosynthesis. In this study, the intracellular level of adenylates of tylosin-producing *S. fradiae* was found to be inversely proportional to the rate of antibiotic synthesis (Fig. 3 and 4).

In batch fermentation of tylosin, the intracellular concentrations of the adenylate nucleotides were observed to drop several fold before the onset of antibiotic biosynthesis. The energy charge was, however, stabilized throughout the fermentation and was in the range of 0.4 to 0.55, which is comparable to that of chlortetracycline-producing *S. aureofaciens* (3). The stabilization of adenylate energy charge involves unknown mechanisms. Dietzler (4) suggested the involvement of adenylate kinase, which preserved the adenylate energy charge at the expense of the adenine nucleotide pool.

Glucose is well known for its depressive effect on many antibiotic-producing systems. Addition of 30 g of glucose per liter in the idiophase

depressed tylosin production, accompanied by a sharp increase in the intracellular adenylate level. Tylosin biosynthesis resumed after a rapid drop in ATP concentration. The adenylate energy charge was, however, practically unchanged. The data obtained suggest that the ATP concentration or the level of the adenylate pool or both may be the intracellular effectors controlling the onset of tylosin biosynthesis. This suggested role of the intracellular adenylates agrees with the results from the time-course study, as discussed previously on the level of the adenylates during the batch fermentation of tylosin.

Propionyl-CoA carboxylase and methylmalonyl-CoA carboxyltransferase are two enzymes involved in carboxylation of propionyl-CoA in tylosin-producing *S. fradiae*. The carboxyltransferase was far more active than the carboxylase; it would probably be the major enzyme in the pathway synthesizing methylmalonyl-CoA or propionyl-CoA. A similar transcarboxylation was reported in *S. noursei* var. *polifungini* (13). In propionibacteria, the carboxyltransferase was found to be more active than the nonspecific carboxylase and it was the most active enzymic constituent of the methylmalonyl-CoA cycle (10).

Bhuwapathanapun and Gray (in press) have studied the stimulatory effect of sodium glutamate on tylosin synthesis. The glutamate dehydrogenase (EC 1.4.1.3) was found active in *S. fradiae* (data not shown), suggesting the importance of the α-ketoglutarate pool in the synthesis of tylonolide precursors. Succinyl-CoA may therefore be the intermediate of the tricarboxylic acid cycle leading to methylmalonyl-CoA and propionyl-CoA and the pool of which would be of importance in the synthesis of these compounds.

During the idiophase, methyloleate was the only carbon source available which would undergo oxidation to acetyl-CoA that would subsequently be utilized for synthesizing tylonolide

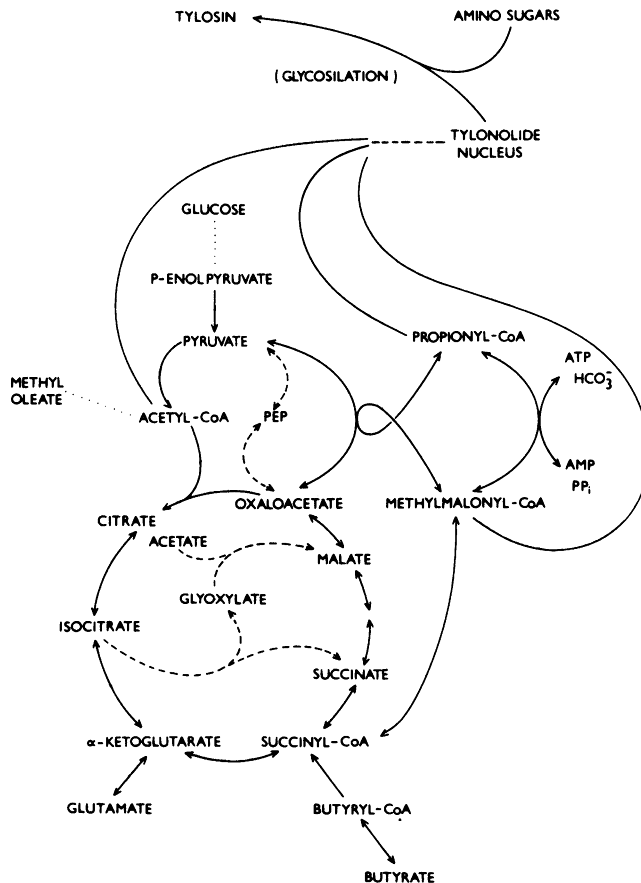


FIG. 9. *Possible metabolic pathway for biosynthesis of tylonolide precursors. Broken lines indicate hypothetical reaction routes.*

precursors. In a recent report, Sprinkmeyer and Pape (17) demonstrated the inhibition of citrate synthase (EC 4.1.3.7) by oleyl-CoA, suggesting that, in the presence of oleyl-CoA, less acetyl-CoA would be metabolized to citrate by condensing with oxaloacetate. If the pool of succinyl-CoA is important in the synthesis of the major tylonolide precursors, it could be therefore plausible to assume the possible involvement of the glyoxylate cycle in the synthesis of acyl-CoA. The suggested pathway, with hypothetical reaction routes, is depicted in Fig. 9.

Glucose appeared to cause a repression effect on the carboxylating enzymes. Addition of glucose to 5-day tylosin-producing cultures reduced the enzyme activities. However, the results obtained do not explain the severe suppression of tylosin synthesis after increased glucose addition. Methyloleate uptake rate was also slowed down as glucose was added in the idiophase. Increased levels of the adenylates were observed after the addition of glucose; this might affect certain biosynthetic enzymes. However, the real mechanism is still obscure at present.

This work suggests the regulatory role of the intracellular adenylates in the control of tylosin biosynthesis. The pathway for the synthesis of tylonolide precursors is also suggested.

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