# Quantitative Microbial Physiology of *Streptomyces coelicolor* A3(2).

by

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A thesis submitted for the degree of Doctor of Philosophy
at the University of Glasgow

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October 1992



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**The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.**

**Anne O. Davidson, October 1992.**

This thesis is dedicated to Mum, and to Dad,
the memory of whom gave me the courage and determination to finish it.

# Contents

# List of Figures and Tables

Chapter 1.



Chapter 2.



Chapter 3.

Chapter 4.

# Abbreviations

| | |
|---|---|
| $dH_2O$ | distilled water |
| RT | room temperature |
| g | gram (or gravity, in reference to centrifugal speeds) |
| l | litre |
| ml | millilitre |
| µl | microlitre |
| mM | millimolar |
| µM | micromolar |
| N | normal |
| PCA | perchloric acid |
| TCA | trichloroacetic acid |
| RNA | ribonucleic acid |
| DNA | deoxyribonucleic acid |
| CHO | carbohydrate |
| psi | pounds per square inch |
| G6P | glucose-6-phosphate |
| PKS | polyketide synthase |
| ORF | open reading frame |
| ACP | acyl carrier protein |
| acetyl CoA/AcCoA | acetyl-Coenzyme A |
| cAMP | cyclic adenosine monophosphate |
| ATP | adenosine triphosphate |
| ppGpp | guanosine 3'-diphosphate 5'-diphosphate |
| pppGpp | guanosine 3'-diphosphate 5'-triphosphate |
| tRNA | transfer ribonucleic acid |
| mRNA | messenger ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| GTP | guanosine triphosphate |
| NMM-J | new minimal medium minus junlon |
| Tris | tris(hydroxymethyl)aminoethane |
| DO | dissolved oxygen concentration |
| CFU | colony forming units |
| G | guanine |
| C | cytosine |
| A | adenine |
| T | thymine |
| U | uracil |
| XMP | purine/pyrimidine monophosphate |
| dXMP | deoxy purine/pyrimidine monophosphate |
| TA | teichoic acid |
| TP | triose phosphate |
| PG | phosphoglycerate |
| PEP | phospho*enol*pyruvate |
| PYR | pyruvate |
| OAA | oxaloacetate |
| OGA/αKGA | oxoglutarate/α-ketoglutarate |
| PFK | phosphofructokinase |
| CMPS | central metabolic pathways |

| | |
|---|---|
| EMP | Embden-Myerhof Pathway |
| PP | pentose phosphate pathway |
| TCA cycle | Tricarboxylic acid cycle / Citric acid cycle |
| $P_i$ | inorganic phosphate |
| PEPC | phospho*enol*pyruvate carboxylase |
| ICDH | isocitrate dehydrogenase |
| ICL | isocitrate lyase |
| NMR | nuclear magnetic resonance |
| MCT | metabolic control theory |
| v/v | volume per volume |
| w/v | weight per volume |
| DF | dilution factor |
| min | minute(s) |
| h | hours |
| TOC | total organic carbon |
| TOCA | Total organic carbon analyzer |
| BSA | Bovine serum albumin |
| HPLC | high pressure liquid chromatography |
| PEG | polyethylene glycol |
| junlon | junlon-110 |

## Acknowledgements

I would like to thank the following people:

Dr. Iain S. Hunter for his supervision of this work and for reading this thesis; Dr. W. Harry Holms for the use of all the facilities in the Bioflux laboratories; Dr. Iain D. Hamilton for many helpful discussions; Dr. David Mousdale for allowing me to use the HPLC.

My time spent in the laboratory at the now non-existent Institut für Mikrobiologie und experimentelle Therapie, Jena (DDR),was very helpful to me scientifically. The experience of an ex-Eastern Block country is unforgettable. I therefore wish to convey my gratitude to Dr. D. Noack for making it possible; to Dr. Martin Roth and Karin Perlet for their help and friendship; to Gerhard and Christine for their friendship and making my visit to Jena very enjoyable. I would also like to thank Lei for making my time in the flat less lonely.

My thanks go to members of the Department of Genetics and Biochemistry who helped me at any time; this includes the ladies on the 3rd floor and the secretaries. Thank-you also to the Strep. group, especially Marion. Most of all, thank-you to Helena and Alison for helping me through the numerous rough patches with their encouragement and friendship. This also applies to the "Bioflux Girls", Margaret and Eileen who helped me enormously and made me laugh.

Finally, I wish to express my sincere and everlasting gratitude to Garry, without whose help and encouragement this thesis would have taken much longer to write.

This work was supported by funding from the SERC (CASE: Bioflux Limited), with supplementary funding from the German Academic Exchange Service and the Stevenson Fund (University of Glasgow).

## Preface

The results section of this thesis consists of three chapters (3, 4 and 5), the figures for which are given at the end of each appropriate chapter.

There are five appendices, A, B, C, D and E, which are located after the final discussion chapter (chapter 6).

## Summary

Production of secondary metabolites by *Streptomyces* requires the use of primary metabolites as precursors. A relationship therefore exists between primary and secondary metabolism which involves diversion of the flow (flux) of these precursors from primary processes to secondary metabolism. Deregulation of control of these fluxes may allow the flux to secondary metabolism to be increased and, ultimately, improve productivity. Attempts to identify enzymes involved in regulation of such fluxes have included the use of "reverse genetics", which has resulted in purification (and cloning of the genes) of several enzymes of the primary metabolic pathways of *Streptomyces coelicolor*. Quantitation of the fluxes to biomass and to antibiotic production would identify additional enzymes of the central metabolic pathways which are involved in diverting these fluxes from primary to secondary metabolism. A straightforward method for quantifying fluxes is that described for *Escherichia coli* by W.H. Holms (1986. Current Topics in Cellular Regulation, **28**: 69-105), which requires derivation of the composition of the micro-organism and knowledge of the central metabolic and biosynthetic pathways. Assuming that primary metabolism of *Streptomyces* was similar to that of *E. coli*, this method was considered a suitable starting point to quantify the fluxes through *S. coelicolor* to biomass and to the polyketide antibiotic actinorhodin. Therefore, a requirement for this was to derive compositional data for *S. coelicolor*.

In a minimal medium suitable for the calculation of carbon fluxes (NMM-J; containing a mechanical dispersing agent), *S. coelicolor* grew in a semi-pelleted fashion in flask and fermenter cultures and exhibited variability of production of both biomass and actinorhodin. Biomass yields were low in comparison to those reported by other workers using different defined media containing carbon-based dispersing agents. Production of actinorhodin occurred in NMM-J when carbon, nitrogen and phosphate were in excess. Dispersed growth of *S. coelicolor* was obtained by continuous cultivation in a phosphate-limited chemostat (in Jena, Germany); single hyphae grew from spores at low dilution rates and branches appeared, grew longer and became entangled as the dilution rate increased. Actinorhodin and undecylprodigiosin were produced simultaneously during the transient period between steady states at each dilution rate, possibly because of metabolic imbalances. A yellow pigment was synthesized during each steady state.

Compositional analyses of *S. coelicolor* involved measurement of the macromolecular, monomeric and elemental contents. An approximate macromolecular composition was determined to be 53% protein, 14% RNA and 6% DNA; the extent of irreproducibility of growth of *S. coelicolor* in NMM-J was reflected in the macromolecular compositions which were variable. The approximate contents of nucleic acids were similar to those reported for *E. coli* at a slow growth rate (*i.e.*, a doubling time of 100 minutes). However, the content of protein was low. Other components were possibly responsible for the lower protein content of the streptomycete in comparison to the enterobacterium. The monomeric composition was determined from the nucleic acid and lipid content and also by direct measurement of amino acids. The amino acid composition showed some relationship to the high (approximately 70%) GC bias of the streptomycete DNA. The elemental composition of *S. coelicolor*, which had a high oxygen content, was similar to those of other *Streptomyces* analyzed. This may have been the consequence of the obligatory aerobic growth of *Streptomyces*.

The monomeric composition of *S. coelicolor* was used to determine the fluxes to biomass using the method of Holms (1986). The fluxes were greatest via pyruvate and least via triose phosphate. Expression as carbon fluxes showed that the highest proportion of the total input flux was to $CO_2$ (approximately 70%), reflecting the low yields of biomass. This theoretical prediction was supported experimentally by measurement of $CO_2$ produced during a single fermentation. This may suggest the requirement for a high maintenance energy. The carbon flux to actinorhodin was less than 2% of the total input, measurement of which was likely to be lost in experimental error. Therefore, production of actinorhodin by wild type strains of *S. coelicolor* may not be suitable for identification of enzymes involved in regulation of secondary metabolism.

Fluxes to biomass and actinorhodin production were compared, assuming organic carbon excreted by *S. coelicolor* was derived from α-ketoglutarate and pyruvate. An increase in flux through phospho*enol*pyruvate (PEPC) was determined which had been reported for stationary phase cells of *Streptomyces* sp. C5 (Dekleva, M. L. and Strohl, W. R. (1988). Canadian Journal of Microbiology, **34**: 1241-1246). Increased activity of PEPC in aging mycelia of *S. coelicolor* had also been observed in this laboratory. It is therefore proposed that PEPC should be concentrated on as a possible candidate enzyme in regulation of production of secondary metabolites.

# Chapter 1

# General Introduction

## 1.1 Introduction

This chapter serves as background information necessary for comprehension of the results chapters in the thesis. In general, it covers three themes concerned with microbial physiology of *Streptomyces*: growth, secondary metabolism and primary metabolism. The first sections deal with why *Streptomyces* are studied and why, in particular, it is important to have physiological studies in addition to the rapidly advancing genetic studies. A second theme covers streptomycete secondary metabolism. It is placed before the theme of primary metabolism because more information is currently available. Some aspects of the biosynthesis of secondary metabolites and regulation of their production are included.

The main focus of this study, however, is on the flow of primary metabolites. Therefore, large sections deal with primary metabolism, its regulation and its relationship to secondary metabolism.

Finally, the aims of the thesis are presented.

## 1.2 Why study *Streptomyces*?

*Actinomycetales* are Gram-positive, soil bacteria which give damp soil its characteristic odour (Ensign, 1978). Present in the order is the group Streptomycetes containing, on the basis of 16S ribosomal RNA (rRNA) similarity, five genera of which *Streptomyces* is the largest (Goodfellow and Cross, 1984). The main reason for interest in *Streptomyces* is their ability to produce metabolites with chemotherapeutic properties. They also exhibit a very complex life cycle. These points are expanded upon in the following two sections.

### 1.2.1 Secondary metabolites - what and why?

Metabolite excretion by micro-organisms has been exploited by man for many centuries, although it was not until the mid 1800's, with the advent of microbiology, that the role of micro-organisms was discovered. Since the 1950's the field of biotechnology has expanded, especially with the discovery of the therapeutic activities of some metabolic products.

The first antibiotic isolated from an actinomycete was actinomycin which was found to act against Gram-positive species (Waksman and Woodruff, 1940). This was closely followed by the discovery of streptomycin from *Actinomyces griseus* (*Streptomyces griseus*), an antibiotic exhibiting properties against both Gram-positive and Gram-negative strains (Schatz *et al.*, 1944). Subsequent discoveries of antibiotics have been numerous, mostly from soil organisms among which production from *Streptomyces* is the most prolific.

Metabolites required for growth are termed primary metabolites and they are defined as being present throughout the life cycle of the organism, have relatively simple structures and are "turned over" by short amphibolic pathways. Products such as actinomycin and streptomycin did not appear in any way to be associated with or necessary for growth, and did not fit the other criteria for primary metabolites. They were therefore called secondary metabolites. Secondary metabolites are defined as not being required for growth, are produced only during the idiophase (the production phase; the growth phase is designated the trophophase), and have complex chemical structures synthesized by long and complicated pathways (Campbell, 1984).

Many roles have been ascribed to secondary metabolites, which are produced by insects and plants in addition to micro-organisms. Functions include insecticides and nematocides, anti-cancer agents and immunosuppressives, mineral scavengers and autoregulators. A comprehensive review of the properties of secondary metabolites is given by Vining (1990).

The reasons why secondary metabolites are produced are not clear at present. Contrary to the idea that they are waste or detoxification products (some secondary metabolites are themselves toxic), evidence suggests that they are important to the organism. The reactions involved in their biosynthesis, which are not present in primary metabolism, must have effected an advantage since the new reactions were costly in energy (Campbell, 1984). The presence of resistance mechanisms in producer-organisms also upholds the view that they are of value (Vining, 1990). These include: modification of ribosomes, modification or replacement of target sites, inactivation or sequestration of the drug molecules, and membrane permeability barriers or efflux mechanisms (Cundliffe, 1989). *Streptomyces* resistance genes are often located in clusters which contain the antibiotic biosynthetic and regulatory genes (Hopwood, 1988). Clustering of

genetic material suggests importance to the organism.

Non-producing organisms also show resistance to certain antibiotics. The induction of penicillinases in some targetted strains by penicillin, may indicate that the production of secondary metabolites is important in the natural environment (Pollock, 1967). This possibly negates the suggestion that secondary metabolites are products of metabolic pathways stimulated during growth in abnormal circumstances, *e.g.*, in submerged culture (Campbell, 1984).

An interesting alternative view to that which claims secondary metabolites are important to the organism, is given by Bu'Lock (1961). He stated that secondary metabolites were not specialised products, but were normal shunt metabolites formed because of an increase in metabolic pool concentration during a period of restricted growth. The synthesis of these metabolites allowed the maintenance of mechanisms which could rapidly return to growth-associated metabolism when conditions became more favourable. The shunt metabolite theory was upheld by Woodruff (1966), although he claimed secondary metabolites conferred no selective advantage on the organism since the extent to which they were produced in nature was much less than in laboratory conditions.

A favoured suggestion as to why secondary metabolites are produced is that they play a role in the survival of the organism, sterilizing the soil at times of nutrient deficiences, thus providing a selective advantage (Katz and Demain, 1977). As described later (section 1.2.2), a differentiation stage occurs during the developmental cycle of *Streptomyces*, resulting in the formation of aerial hyphae. Nutrients for growth of aerial hyphae are provided by lysis of the vegetative mycelium. It is, therefore, a period of vulnerability. Antibiotic production can take place at this time (Mendez *et al.*, 1985), possibly to protect the surrounding area from bacterial "predators" (Chater and Merrick, 1979). The presence of pleiotropic switches (effector molecules involved in both differentiation and secondary metabolite production) also suggests antibiotics are produced for protection and to ensure the continuation of the species (Hopwood,1988).

The evolution of secondary metabolites is also under speculation. They are unlikely to have been produced unless they conferred a functional evolutionary advantage upon the organism (Katz and Demain, 1977; Stone and Williams, 1992). Synthesis may have occurred initially due to random

mutations in metabolic genes with selectional advantage allowing maintenance of the new biosynthetic genes (Stone and Williams, 1992). Clustering of the genes may have enabled the advantage to be passed on to a closely related species.

In contrast, Davies (1990) believes antibiotics may have been pre-biotic effector molecules which maintained their binding capacities for nucleic acids and protein when replaced by polypeptides. It was proposed that they took on their modern functions during the course of evolution. However, the hypothesis does not explain the evolution of the modern antibiotic biosynthetic pathways (Stone and Williams, 1992).

### 1.2.2 Developmental cycle of *Streptomyces*.

*Streptomyces* exhibit a complex growth cycle with differing morphological states (figure 1.1). These states are described below.

i) Germination. In general, germination of a streptomycete spore occurs under favourable environmental conditions, although some spores require to be activated, *e.g.* by heat shock (Ensign, 1978). On germinating, the spores swell followed by the emergence of one or more germ-tubes, the walls of which originate from a pre-existing inner layer of the spore wall (Sharples and Williams, 1976). The initial stages of germination require the presence of divalent cations (studies have revealed the presence of a calcium-dependent ATPase (Stoxen and Ensign, 1991)) and energy, presumably provided by the catabolism of an endogenous energy reserve (Eaton and Ensign, 1980; Hardisson *et al.*, 1978). A possible candidate molecule for the reserve is trehalose (Ensign, 1978; Salas *et al.*, 1984) which is rapidly hydrolized on initiation of germination (McBride and Ensign, 1990). During this period, the Embden Myerhof Pathway is highly active, with the TCA cycle and Pentose Phosphate pathway providing precursors for the biosynthesis of macromolecules (Salas *et al.*, 1984). Protein and stable RNA synthesis occurs simultaneously with spore swelling (Guijarro *et al.*, 1982), while DNA synthesis coincides with the later emergence of germ-tubes (Hardisson *et al.*, 1978).

ii) Vegetative hyphae. Development of the germ-tubes results in the formation of young vegetative hyphae which grow radially, by means of branching, either along the surface of the growth substrate or through it

(Locci and Sharples, 1984). *Streptomyces* mycelia, like fungal mycelia, are non-fragmenting and exhibit a monocentric growth pattern (Locci, 1976; as cited by Locci and Sharples, 1984). Elongation occurs by apical hyphal extension (Brana *et al.*, 1982) through the addition of newly synthesized wall material (Gray *et al.*, 1990a). Branching occurs after apical extension, but no branches are formed in old parts of the mycelium (Schuhmann and Bergter, 1976; as cited by Locci and Sharples, 1984).

The kinetics of growth are similar to those of filamentous fungi: total mycelial length and number of branches of *S. coelicolor* increase at the same exponential rate (Allan and Prosser, 1983). However, germ tubes and the first branch hyphae show linear extension, an observation not seen in fungi (Trinci, 1971a) or in some other streptomycete species, *e.g.*, *S. hygroscopicus* (Schuhmann and Bergter, 1976; cited by Allan and Prosser, 1983) and *S. granaticolor* (Kretschmer, 1982). Colony extension on solid media is linear (Allan and Prosser, 1985) and like fungi, the colonies exhibit a peripheral growth zone which contributes to the exponential production of biomass, while individual hyphae extend at a constant linear rate (Trinci, 1971b). Hyphae can therefore grow beyond regions of nutrient limitation. This growth pattern is advantageous to an organism which can break down and metabolize insoluble organic material (Chater and Merrick, 1979).

Cross walls, classed as Type I (Williams *et al.*, 1973), are formed in vegetative mycelia by displacement of an annulus of wall material. This results in septa twice the thickness of the mycelial wall. Individual cells have a typical prokaryotic organization (Locci and Sharples, 1984) and communication between adjacent cells occurs by means of microplasmodesmata (Strunk, 1978). Unlike eubacteria, the cross walls of *Streptomyces* do not normally split on cell division.

iii) Aerial hyphae. The extension of individual hyphae from the vegetative mycelium allows spatial and temporal differences to occur within the colony: new hyphae may be formed at a time when older parts of the mycelium are undergoing a process of differentiation. Differentiation occurs during periods of low nutrient availability, manifested by the production of vertically growing hyphae. These hyphae are known as aerial hyphae and arise as branches from the uppermost vegetative mycelium (Hopwood, 1960). They differ from vegetative hyphae in that they are straighter and branching occurs much less frequently. They are also covered with a fibrous sheath (Rancourt and Lechevalier, 1964) and exhibit

hydrophobic properties (Higgins and Silvey, 1966).

Aerial hyphae formation has been associated with storage compound accumulation and also autolysis by vegetative mycelia. A model has therefore been proposed for the emergence of such aerial hyphae involving cytoplasmic osmotic potential (Chater, 1989). According to the model, degradation of storage compounds would result in increased turgor pressure within the vegetative cells which could be relieved by production of aerial hyphae from weakened points in the hyphal walls. Extension of these hyphae would occur into the air because of the hydrophobic properties of their surfaces which could be formed from specialized molecules excreted by surface mycelia at the air-hyphal interface. The formation of these aerial hyphae, which can exist in a non-aqueous environment, is unique amongst prokaryotes but could provide selective advantage in a terrestrial environment (Chater and Merrick, 1979).

iv) Sporulation. Septation also occurs in aerial hyphae, in a manner similar to that in vegetative hyphae (Wildermuth and Hopwood, 1970), prior to the onset of another process of differentiation, sporulation. Initial steps in sporulation involve coiling of the apical cells and division into spore-sized compartments by sporulation septa (Wildermuth, 1970; Wildermuth and Hopwood, 1970). Sporulation septa of *S. coelicolor* differ from cellular septa. They may be classed into three types (Hardisson and Manzanal, 1976) but, in general, are formed synchronously, at regular intervals, over a certain length of hyphae. Adjacent layers are separated by an obvious gap containing "deposits" which are possibly degraded during spore maturation (Brana *et al.*, 1981). Spore walls are constructed by the addition of material to the septum and the inner side of the parental hyphal wall. As the spores mature, their walls thicken and the fibrous sheath rounds off to permit separation (Wildermuth and Hopwood, 1970).

A reliable indication of the physiological age of a *Streptomyces* colony grown on solid medium, is the colour (Wildermuth, 1970). Formation of aerial mycelia is signified by change in the shiny appearance of the vegetative mycelia to white and "powdery". As sporulation occurs, the aerial mycelia turn grey with spore chains, the colour darkening as the spores mature.

## 1.3 Streptomycete genetics versus physiology

From the previous section it can be concluded that *Streptomyces* are very complex organisms, but, to date, in comparison with an organism such as *Escherichia coli*, very little is known about many of their processes. Most studies have been carried out by genetic analysis, including characterization of the chromosome and natural plasmids, identification of effector molecules involved in differentiation and elucidation of genes concerned with secondary metabolism (Chater and Merrick, 1979; Chater and Hopwood, 1984; Hunter and Baumberg, 1989). Of the genus, *S. coelicolor* A3(2) is typical with respect to streptomycete genetics and has thus become the most genetically well-characterized species (Hopwood *et al.*, 1973; Hopwood, 1988).

Physiological studies of *Streptomyces* have, however, not matched advances in genetics (Hobbs *et al.*, 1989) which have been rapid because of use of molecular genetic techniques. Analysis of growth and metabolism of an organism requires cultivation in minimal media. One reason why *E. coli* emerged as the organism of choice for genetic and physiological studies in the 1930's was because of the ease by which it could be grown in minimal media (Schaechter and Neidhardt, 1987). The filamentous growth habit of *Streptomyces* poses problems in minimal media with the formation of tight spherical structures (sections 1.5.3; 3.1; Williams *et al.*, 1974).

Enhancement of production of antibiotics in the fermentation industry has relied on classical genetic techniques such as strain selection and mutagenesis. For example, penicillin production has increased 10,000 fold since the 1940's (Hersbach *et al.*, 1984). In addition, recent use of molecular genetics has resulted in a 30-fold excess in antibiotic production from a recombinant of *S. coelicolor* containing a plasmid encoding the antibiotic gene cluster (Malpartida and Hopwood, 1984; cited by Hunter and Baumberg, 1989). However, present production levels of penicillin are still only 40% of what could be produced (Pirt, 1987), converted from only 6% of the carbon source (Cooney, 1979). The use of microbial physiology is therefore necessary for further improvements, both to examine the environmental influence on gene expression (Pirt, 1987) and to minimize wastages in the fermentation (Holms *et al.*, 1991).

## 1.4 Microbial Physiology - a definition

Microbial physiology is the study of the function and structure of micro-organisms. It encompasses a wide range of topics including cell structure and the dynamic aspects such as growth, metabolism and genetics, *i.e.*, microbial physiology examines the cell as a whole. Metabolism of micro-organisms is mainly studied under the separate heading of biochemistry, while microbial genetics has expanded to such a degree that it is no longer regarded as part of microbial physiology. The techniques and principles of genetics have, however, aided in the advancement of information on both structure and metabolism (Moat and Foster, 1988).

Many publications are available concerning microbial physiology which describe the subject in varying depths (*e.g.*, Dawes and Sutherland, 1976; Moat and Foster, 1988; Neidhardt *et al.*, 1990). The latest publication (Neidhardt *et al.*, 1990) takes a molecular approach and expands on the commonly used knowledge of enteric bacteria (*e.g.*, Neidhardt *et al.*, (eds.), 1987) by giving a panoramic view covering numerous microbial species. In many studies *Escherichia coli* has been regarded as a typical example of a bacterial cell. As mentioned previously (section 1.3), from the 1930's *E. coli* emerged as the experimental organism for physiological and genetic studies (see Schaechter and Neidhardt, 1987, for a synopsis on the magnitude of information obtained from *E. coli*). The biochemical and genetic analyses of *E. coli* and *Salmonella typhimurium* thus far outweigh studies performed with other micro-organisms (Dawes and Sutherland, 1976).

Quantitative microbial physiology is concerned with measurement of cellular metabolism and its products. Initial quantitative studies on *E. coli* were carried out by Taylor (1946) who, on studying the relationship between *E. coli* and the bacteriophage T2, measured the carbon, nitrogen and phosphorous content of both host and virus. Measurement of the phosphorous in an acid-soluble fraction of the cellular biomass resulted in a basic determination of the nucleic acid content. Later, Roberts *et al.* (1955) reported their comprehensive studies of the biosynthetic pathways of *E. coli* which included measurements of polymeric (macromolecular) and monomeric (building block) components. The techniques of using radioactive tracers with chromatography allowed the elucidation of the biosynthetic relationship between certain monomers, *e.g.*, the aromatic amino acids. This work has provided the foundation upon which subsequent analyses have been based (*e.g.*, Umbarger, 1977; Holms, 1986;

Neidhardt, 1987).

## 1.5 Microbial growth

Growth of the micro-organism is a key element in physiological studies. In order to examine growth and metabolism, two techniques have been developed for cultivation in liquid medium: batch and continuous culture.

### 1.5.1 Batch culture

Batch cultivations are partially closed systems where the only exchange of matter is by gaseous exchange. Conditions inside a vessel are transient since biomass increases as nutrients are continually utilised and products are secreted. Limitation of growth is caused by an inevitable reduction in concentration of a limiting nutrient.

Batch cultivations are described as having four distinct phases:

i) lag phase. This is a period of adjustment during which cells adapt to a new environment and is thus a time of zero growth. It is generally observed when stationary phase cells are transferred into fresh medium. Growing cells have a different chemical composition to those in stationary phase (see ii and iii of this section), therefore stationary phase cells must undergo a change in composition before they are capable of initiating growth. Lag phase is also observed when cells are removed from a complex medium and placed into a minimal medium. This process is known as nutritional down-shift and is associated with differential changes in macromolecular content. During this time, genes, repressed because of the availability of the nutrients in the complex medium, are ultimately derepressed. Sporulating bacteria inoculated as spores into the medium also exhibit a lag phase, during which germination occurs.

An event also associated with nutrient limitation or removal of amino acids from the medium is the Stringent Response (Cashel and Rudd, 1987). This response is observed to occur due to limitation of one or more amino acids, for example during a shift from rich to poor media, or to exhaustion of a carbon source. It is manifested by a decrease in RNA accumulation, DNA replication, and biosynthesis of several metabolites. It is also

associated with a marked increase in ppGpp synthesis. The signal for ppGpp synthesis, from guanosine diphosphate (GDP) or guanosine triphosphate (GTP), was found to be binding of an uncharged transfer RNA (tRNA) to the ribosome resulting in activation of a ribosome-bound phosphotransferase, RelA (or (p)ppGpp synthetase I). ppGpp affects GTP-requiring reactions such as initiation and elongation of translation. It has also been shown to specifically inhibit rRNA, and mRNA for ribosomal proteins and may regulate transcription by binding to RNA polymerase. Mutations in *relA* resulted in decreased accumulation of ppGpp during amino acid limitation, with no inhibition of RNA synthesis; *relA* mutants are known as relaxed mutants. Another enzyme involved in ppGpp turnover is SpoT (ppGppase). A *spoT* mutant exhibited increased ppGpp levels resulting in slight slowing of growth. The stringent response is, therefore, a sensing mechanism which adjusts the synthesis of protein synthesizing machinery depending on conditions, and enhances cellular viability by conserving energy required for ribosome synthesis.

ii) exponential, or growth, phase. If nutrients are available in excess, unicellular bacterial cell weight increases in an exponential fashion which is accompanied by an exponential increase in macromolecules. This results in the maintenance of a constant chemical composition and may be a time of balanced growth (Campbell, 1957): it is the most reproducible state of a culture (Neidhardt *et al.*, 1990). However, excretion of metabolites - proteins, cell-wall associated molecules, overflow products and lytic products - can also occur at this time in some cases (Wanner and Egli, 1990).

The rate of change of biomass concentration, dx/dt, is described by the equation:

$$\frac{dx}{dt} = \mu x$$

where $\mu$ is the specific growth rate, and x is the biomass concentration. During exponential phase, $\mu$ is at a maximum and is constant, the level depending on the medium composition (Schaechter *et al.*, 1958). $\mu$ eventually becomes limited by the exhaustion of an available nutrient or by the accumulation of toxic products of metabolism. The cells then enter a transition period between growth and stationary phases, during which macromolecular components are synthesized at unequal rates resulting in unbalanced growth. This period has not been observed experimentally and is thought, therefore, to be quite short (Wanner and Egli, 1990).

iii) stationary phase. The proportion of actively growing cells declines during this period. A reduction in the rate of macromolecular synthesis occurs, but initiated rounds of DNA replication and cell division are completed; cell size therefore decreases (Neidhardt *et al.*, 1990). DNA precursors may be formed from degradation products of firstly RNA and then protein (Mandelstam *et al.*, 1982). Possible recycling of the limiting factor can occur because of lysis, and metabolites produced during growth phase are utilized. These metabolites include acetate produced by *E. coli*, (Andersen and von Meyenburg, 1980), and ethanol, a product of the Crabtree effect of alcoholic fermentation by yeast which occurs in the presence of glucose under strictly aerobic conditions (van Dijken and Scheffers, 1986). However, there is no net growth since growth is counterbalanced by death. Carbon and energy reserves may be stored at this time, or, in species which undergo a complex life cycle, e.g., *Bacillus* or *Streptomyces*, developmental changes may occur.

iv) death phase. This results from a depletion of cellular energy reserves, and biomass concentration decreases. However, it is debated as to whether there is a specific death phase. In nature, nutrient availability is generally limited and a state of starvation in micro-organisms is therefore usual (Morita, 1988). Complex morphological species have a survival strategy called sporulation, and it is possible that non-spore-forming bacteria may also undergo a process of molecular realignment. During carbon starvation (and also phosphate and nitrogen starvation), *E. coli* synthesises proteins not synthesized during growth (Groat *et al.*, 1986), precursors for which may be provided by degradation of growth-related proteins (Mandelstam, 1960; Reeve *et al.*, 1984). Sequential expression of carbon starvation (*cst*) genes, similar to that of resistance genes in *Bacillus*, also occurs at this time in *E. coli* (Groat *et al.*, 1986), and specific loci (*sti* genes) have been found recently to be important for starvation-survival in *Salmonella typhimurium* (Spector and Cubitt, 1992). Reviews of survival mechanisms in *E. coli* are given by Matin *et al.* (1989) and Kolter (1992).

Examination and elaboration of the kinetics of batch cultures are complicated because of the transient nature of the parameters. Nutrient uptake and cellular growth rate cannot be controlled in batch cultures. In continuous cultures, however, growth rate can be held constant, allowing the growth of an organism in a metabolic steady-state, in an unchanging environment.

### 1.5.2 Continuous culture

The advantage of continuous culture is the constancy of growth rate and nutrient concentration, with the possibilities to manipulate variables externally. The method has not been widely used, however, because of the difficulty in maintaining sterility over a long time period, and the probability of the appearance of mutants.

Monod (1950, as cited by Herbert *et al.*, 1956) and Novick and Szilard (1950), first described the system of continuous cultivation. Six years later, in response to objections to the theories, Herbert *et al.* (1956) carried out an extensive theoretical and experimental study based on the fundamental principles described in 1950. Continuous cultivation is an open system with exchanges of nutrients, cells and gases. Medium is pumped into a vessel at a flow rate, f, and, together with growing cells, leaves the vessel at the same rate. This results in a constant volume inside the vessel, maintained by means of a weir. Effluent culture may be pumped out, or ejected by a pressure build up inside the vessel, via the air outlet tubing. Agitation is necessary for the uniform distribution of medium entering the vessel.

Residence times of molecules in the culture are described as the ratio:

$$\frac{f}{v} = D$$

D is the dilution rate, and, if greater than the rate of formation of biomass, wash-out occurs. Wash-out rate is expressed as:

$$\frac{-dx}{dt} = Dx.$$

The growth rate of the biomass is represented in a similar way to that in the exponential phase of batch culture. In batch cultivations, $\mu$ is a maximum since all nutrients are in excess. In continuous culture, however, according to Monod (1942, as cited by Herbert *et al.*, 1956), $\mu$ is proportional to the concentration of the limiting substrate, s, at low values of substrate concentration and reaches saturation at high values. This is shown by the equation:

$$\mu = \mu_m \frac{s}{k_s + s}$$

where $\mu_m$ is the growth rate constant at saturating levels of s, and $k_s$ is equivalent to the substrate concentration when $\mu = 0.5\mu_m$. Exponential growth can, theoretically, thus occur from $\mu = 0$ to $\mu = \mu_m$, if the substrate concentration is held at the appropriate value (Herbert *et al.*, 1956).

A net rate of increase of biomass must take into account both the growth rate and the dilution rate and is expressed simply as:

$$\text{increase} = \text{growth} - \text{output}$$

$$\frac{dx}{dt} = \mu x - Dx.$$

If $\mu > D$, biomass concentration increases; if $\mu < D$, wash-out of the culture occurs. If $\mu = D$, however, the rate of increase of biomass is zero and x is constant. This condition is known as steady-state. The initial steady-state, following inoculation, is reached because the substrate concentration falls with increasing biomass concentration. The reduction in s ultimately causes a reduction in $\mu$ which eventually becomes equal to D. Increasing D increases the availability of s and $\mu$ increases until s is again reduced, and $\mu = D$. Continuous cultivation is, therefore, an essentially stable system where any alteration in D results in an automatic readjustment of the system.

### 1.5.3 Growth of *Streptomyces* in submerged culture

As described in section 1.2.2, *Streptomyces* exhibit a filamentous growth pattern similar to that of fungi. Kretschmer *et al.* (1981) formulated equations to describe the growth of actinomycete species, parameters of which, when expressed as a ratio, represented the fungal hyphal growth unit, *i.e.*, hyphal length per tip in the mycelium (Trinci, 1974). They found that, in continuous culture, at low dilution rates, branching in *S. hygroscopicus* was diminished, possibly reflecting preferential growth of apical hyphae under poor nutrient conditions. Higher growth rates resulted in derepression of branching, perhaps to optimise nutrient uptake from the locality. The branching frequency of *Aspergillus nidulans* has been shown to be related to growth rate (Katz *et al.*, 1972). It is possible, therefore, that streptomycete mycelia could be of two morphologies in batch culture: one highly branched, formed during nutrient excess, and one straight with few branches. Consequently biomass is unlikely to follow exponential growth

kinetics (Bushell, 1988).

Branch initiation may be regulated by a mechanism involving the physical condition of the biomass of filamentous organisms (Trinci *et al.*, 1990). The hyphal radius of both fungal and streptomycete species increases with growth rate whereas the hyphal growth unit length decreases while the hyphal growth unit volume remains constant.

Although Bader (1986) warned against comparing prokaryotic and eukaryotic organisms, the similarities in streptomycete and fungal growth, and the lack of knowledge of *Streptomyces* physiology allows the use of fungal systems as growth models. One area common to both organisms is pellet formation in submerged culture.

Fungi exhibit two growth forms in liquid culture, filamentous and pelleted (stromatic; Pirt, 1966). The filamentous form comprises a tangled mass of hyphae with uniform exposure to the medium. Pellets, however, are composed of a central, compact mass of tightly interwoven hyphae surrounded by a less dense outer layer. The density of the centre limits diffusion of nutrients and oxygen, causing an anaerobic microenvironment in which the hyphae cease to grow. Continued growth of peripheral hyphae, however, leads to physiological differences in cells throughout pellets which exhibit cube root growth kinetics. Pirt (1966) developed algebraic equations showing that the thickness of growing peripheral shells was dependent on the rate of diffusion of the medium into the pellet. In a pellet of 6mm diameter, however, microprobe measurements detected only very low concentrations of dissolved oxygen at 100μm into the periphery; no oxygen was present at 135μm (Huang and Bungay, 1973).

*Streptomyces* also exhibit pelleted growth in liquid media (Williams *et al.*, 1974) resulting in non-homogeneous cultures unsuitable for physiological studies. Formation of pellets can be a result of a low concentration of spore inoculum (Smith and Calam, 1980), or conidia (Camici *et al.*, 1952), which are not subjected to high competition for nutrients and therefore branch frequently. Higher inoculum concentrations may lead to less dense pellets or filamentous growth which increases the viscosity of the culture and reduces oxygen mass transfer coefficients (Bader, 1986). However, oxygen transfer may take place by means of trapped bubbles or eddies in the system.

Morphological differentiation is an infrequent event during growth of

*Streptomyces* in liquid culture and depends on culture conditions. For example, sporulation was seen to occur in cultures of several species of *Streptomyces* but only after a period of nutritional downshift and when phosphate was exhausted (Daza *et al.*, 1989). However, sporulation of *S. venezuelae* was observed in nitrogen limited cultures containing excess phosphate, but the differentiation process was subject to carbon catabolite repression (Glazebrook *et al.*, 1990).

Continuous cultivation of *Streptomyces* has been studied for many years. Sikyta *et al.* (1961) grew *S. aureofaciens* in a synthetic medium in continuous culture and observed the occurrence of non-filamentous growth at high dilution rates, in contrast to the filamentous growth of lower dilution rates; later results with *S. hygroscopicus* were consistent with this (Kretschmer *et al.*, 1981; cited by Bushell, 1988). At low dilution rates, the amount of *S. aureofaciens* biomass decreased simultaneously with a cessation in chlortetracycline production (Sikyta *et al.*, 1961). Pellets formed in batch cultures of *S. tendae* were permanently removed on switching to continuous culture and growth remained filamentous (Lohr *et al.*, 1989). Dispersed mycelia are a requisite for continuous cultivation of filamentous organisms (Kretschmer *et al.*, 1981; cited by Lohr *et al.*, 1989).

Difficulties have been found in trying to grow *Streptomyces* at high growth rates (Bushell, 1989). This may be attributed to increased susceptibility of highly branched forms, found at higher dilution rates, to hyphal damage as observed with *S. cattleya* (Bushell, 1988). Antibiotic production is affected by hyphal breakage because of decreased cellular viability. This may result in decreased productivity at high dilution rates (Bushell, 1989).

The most suitable cultures for industrial fementations and physiological studies are dispersed and growing exponentially. Methods for achieving dispersed growth in submerged cultures of *S. coelicolor* are described in section 3.1.

### 1.5.4 Control of growth rate

A theory concerning regulation of branch initiation in fungi, and therefore growth rate, has been previously mentioned (section 1.5.3). It is possible that the physical state of streptomycete biomass may also be involved in control of growth rate. $Ca^{2+}$ ions and cAMP are also reported to have a role in

regulation of fungal hyphal elongation and branch formation (Trinci *et al.*, 1990). However, although cAMP is present in *Streptomyces* (section 1.7.1), it is unlikely that the cyclic nucleotide is involved in such a signal transduction mechanism as in eukaryotes. What mechanisms are therefore likely to be associated with control of growth rate in *Streptomyces*?

Early studies on *E. coli* revealed that medium composition and, therefore environmental conditions, affected the rate of growth (Schaechter *et al.*, 1958), which, in turn, influenced cellular composition (Bremer and Dennis, 1987). Cells grown at increasing growth rates from $0.6h^{-1}$ to $2.5h^{-1}$ were observed to have differing relative amounts of macromolecules. RNA exhibited the greatest relative change, becoming a much higher proportion of biomass. The high RNA content was shown to be due to an increase in concentration of ribosomes which was required for a higher rate of protein synthesis: because bacteria do not always grow at their maximum rates, maintenance of a precisely set maximum amount of protein synthesizing machinery would be costly in energetic terms (Neidhardt *et al.*, 1990). How then is synthesis of this machinery regulated with respect to growth rate?

An initial theory concerning regulation of total RNA synthesis was ribosomal protein feedback inhibition (as reviewed by Nomura *et al.*, 1984). Ribosomal protein genes are present in operons and it was suggested that when ribosomal protein concentration exceeded that of ribosomal RNA (rRNA), a certain protein from each operon would bind to specific sequences on the messenger RNA (mRNA). However, recently it has been found that autogenous control of the S10 operon by the S4 ribosomal protein was possibly necessary but not sufficient for growth rate dependent regulation (Lindahl and Zengel, 1990). This indicates the requirement for additional regulatory processes.

Experimental evidence is gathering for a growth rate regulation mechanism mediated by guanosine 3'-diphosphate, 5'-diphosphate (ppGpp). ppGpp has been found to be involved in growth rate control of stable RNA synthesis (Ryals *et al.*, 1982). Since ppGpp has been shown to have a major role in the bacterial stringent response (section 1.5.1), it was suggested that there are only arbitrary distinctions between growth rate control and stringent control in *E. coli* (Baracchini and Bremer, 1988). In addition, studies on DNA replication suggested ppGpp may play a role in control of DNA synthesis (Chiaramello and Zyskind, 1990), and activity of an rRNA promoter, *rrnB* P1, has been shown to be stringently controlled (Hernandez and Bremer,

1990). However, Nachaliel *et al.* (1989) showed that proteins associated with the 30S ribosomal subunit of *E. coli* were able to bind to the activator sequence of the rRNA promoter, *rrnA* P1, and demonstrated that there was a correlation between binding activity and growth rate. Although ppGpp inhibited binding of such proteins, the nucleotide was shown not to be directly involved in growth rate control. Other studies have suggested ppGpp may not be involved in growth rate regulation at all, since *relA* and *spoT* mutants, devoid of ppGpp synthesizing pathways, were relaxed for stringent control but the growth rate control of rRNA was unaffected (Gaal and Gourse, 1990).

Alternatively, control of growth rate in bacteria may be mediated by the intracellular concentration of building blocks (monomers, *i.e.*, amino acids, nucleotides, etc.). Algebraic models have been formulated to show that concentrations of monomers were related to the rate of RNA synthesis and were thus involved in the regulation of growth rate via effector molecules (Bleecken, 1988; 1989). The models were based on the theory that cells monitor monomer production rates. Another model proposed by Marr (1991) also suggested that monomer concentration may be involved in growth rate control via sensing by and control of macromolecular biosynthesis (Jensen and Pederson, 1990).

*Streptomyces* exhibit a stringent response analogous to that of *E. coli* (section 1.7.2) but the resulting increased concentration of ppGpp was found to be 110 fold less than that of the enteric bacteria (Riesenberg *et al.*, 1984). It remains to be seen whether feedback inhibition of ribosomal protein synthesis, ppGpp or monomer concentration are involved in streptomycete growth rate control.

## 1.6 Secondary metabolism in *Streptomyces*

Knowledge of metabolic processes in *Streptomyces* is still very limited. The use of molecular genetics has allowed the characterization of several genes, mostly those, however, involved in the regulation of differentiation and antibiotic production, and in antibiotic biosynthesis. Because of the isolation and characterization of these gene complexes, more information is available concerning secondary metabolism than primary metabolism. Therefore some aspects of secondary metabolite production will be considered first.

Antibiotic production by most *Streptomyces* usually takes place at cessation of growth, or simultaneously with growth at very low growth rates. Expression of the biosynthetic genes for these antibiotics is at a maximum during the transient period between growth and stationary phases (Behal, 1986). Enzyme activities are at their highest at this time, then decrease (Hunter and Baumberg, 1989). For example, activity of the *O*-methyltransferase involved in the final step of puromycin biosynthesis decreases rapidly possibly due to enzyme inactivation (Pogel, 1975), whereas synthesis of arylamine synthetase stops at a certain concentration of chloramphenicol in cultures of *S. venezuelae* (Jones and Westlake, 1974). Feedback inhibition also regulates the synthesis of the enzymes for tetracycline formation (Votruba and Behal, 1984). Expression of resistance genes coincides with that of the biosynthetic genes, as shown by the sensitivity of antibiotic-producing species to their own antibiotics during growth (Martin and Demain, 1980).

Although several antibiotic gene clusters have been cloned from *Streptomyces* (Hunter and Baumberg, 1989), to date not all the gene products have been assigned functions. The chemical diversity of secondary metabolites would perhaps indicate the presence of hundreds of different enzymic activities throughout the streptomycete genera but this is not the case. Antibiotics may be classified into a few groups, each containing similar biochemical pathways (Seno and Baltz, 1988); genetic homology has been found between certain biosynthetic pathways in these groups (Malpartida *et al.*, 1987a; 1987b). They also share only a small number of metabolic intermediates as precursors (Martin and Demain, 1980). The following section describes what is presently known about the biosynthesis of some antibiotics in each major group, and other ungrouped products.

### 1.6.1 Polyketide antibiotics.

In nature, polyketides range from plant products used as food flavourings to iron uptake molecules. A review describing the synthesis and molecular genetics of polyketide products is given by Hopwood and Sherman (1990). In general, as first proposed by Birch (1967), polyketides are biosynthesized by mechanisms similar to those involved in the formation of fatty acids, the precursor for which is acetyl Coenzyme-A (acetyl-CoA). A simple example is given by the biosynthesis of the unbranched, saturated fatty acids, maximum length $C_{18}$ (stearic acid). Acetyl-CoA undergoes transacylation

onto the terminal serine of the phosphopantetheine arm of an acyl carrier protein (ACP). The extender unit, malonyl-CoA, formed by the carboxylation of acetyl-CoA, binds to the ACP and reacts with acetyl-ACP forming acetoacetyl-ACP in a condensation reaction. The β-keto group is removed by a process of reduction (giving a hydroxyl), dehydration (an enoyl) and further reduction (figure 1.2). Extension of the fatty acid chain occurs by successive additions of two carbon units in the same manner (Lynen, 1972).

Polyketide synthesis differs from that of fatty acids in that one or more of the reactions may be carried out at different steps resulting in molecules containing keto, hydroxyl, enoyl or alkyl functionality at specific points (figure 1.2). Determination of functionality has been suggested to occur at each specific stage, not on completion of the chain (Hutchinson *et al.*,1988). This is shown by incorporation of suitably modified intermediates into erythromycin (Cane and Yang, 1987) and into a polyketide precursor of tylosin (Yue *et al.*, 1987). Further evidence is given by the isolation of polyketide precursors from mutants blocked in the pathways of mycinamicin (Kinoshita *et al.*, 1988), tylosin (Huber *et al.*, 1990) and tetracenomycin C (Yue *et al.*, 1986). These precursors were shown to have chemical structures similar to those found in the products. Diversity in polyketide structure and function is also obtained by use of different starter and extender units (*e.g.*, propionate or butyrate) and, once the chain is assembled, by the formation of aromatic, ether or macrolide rings and addition of methyl groups, terpene chains or sugar residues (Hopwood and Sherman, 1990).

The similarity of formation of fatty acid and polyketide chains suggests that the synthesizing enzymes have similar properties. Fatty acid synthases (FASs) are classed into two types, type I and type II. The bacterial FAS complex is type II and is composed of separate polypeptides for the different reactions in addition to the ACP (Lynen, 1972). Acetyl and malonyl transferases are also involved. Type I FASs are found in eukaryotic cells and may have evolved by fusion of genes encoding the polypeptides of type II FASs (McCarthy and Hardie, 1984). Vertebrate FASs are multi-functional enzymes with domains expressing the activities found in bacterial FAS polypeptides, including an ACP domain. Fungal FASs comprise two subunits, α and β, which in *Saccharomyces cerevisiae* are trifunctional and pentafunctional respectively, arranged as $\alpha_6\beta_6$. The FAS of *Penicillium patulum* is very similar to that of *S. cerevisiae* (Wiesner *et al.*, 1988),

however biosynthesis of the polyketide, 6-methylsalicylic acid, by *P. patulum* is catalysed by a polyketide synthase (PKS) type I, resembling vertebrate FASs (Beck *et al.*, 1990).

Actinorhodin is a polyketide antibiotic produced by *Streptomyces coelicolor* (see later in this section). Protein sequences deduced from open reading frames (ORFs) in the *actI* gene of *S. violaceoruber* Tü 22 show high similarity (Sherman *et al.*, 1989) to the *E. coli* FAS enzyme, β-ketoacyl synthase I (FabB condensing enzyme; Cronan and Rock, 1987. Two additional condensing enzymes, FabF and FabH are also present in *E coli.*, and each has a slightly different function (Tsay *et al.*, 1992)). The *actIII* gene of *S. coelicolor* is assumed to encode a β-ketoreductase (Hallam *et al.*, 1988), showing such an activity during biosynthesis of anthraquiniones and anthracyclines when introduced into *S. galilaeus* (Bartel *et al.*, 1990a). An additional ORF in the actinorhodin gene cluster is postulated to encode a bifunctional cyclase/dehydratase (Hopwood and Sherman, 1990). Since *actI* and *actIII* genes have been found to be similar to polyketide synthase genes in other species (Malpartida *et al.*, 1987a), streptomycete PKSs have been deemed to be type II.

Streptomycete ACPs have also been shown to have amino acid sequences homologous to enterobacterial type II ACPs and to streptomycete FAS ACPs (Hale *et al.*, 1987; Revill and Leadley 1990). However, conformational differences exist between the ACP of the *S. glaucescens* PKS and the *E. coli* and *S. glaucescens* FAS ACPs, as detected immunologically (Shen *et al.*, 1992). Nevertheless, organization of PKS enzymes into such a type II complex removes the possible rate-limiting factor of the necessity of substrates to diffuse from enzyme to enzyme if these enzymes were in different cellular compartments (Behal, 1986).

Recently, an ORF in the erythromycin gene cluster of *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*; Labeda, 1987) has been shown to encode a large protein (molecular weight, 332,427; Cortes *et al.*, 1990). Nine sections of the amino acid sequence were similar to the catalytic activities of FASs and PKSs and the C-terminal sequence of a second ORF was ACP-like. Donadio *et al.* (1991) subsequently showed that the gene cluster was divided into six repeated modules, all encoding FAS-like functions with one ORF containing two modules. A model was therefore formed for the biosynthesis of the polyketide moiety of erythromycin with one elongation step per module (figure 1.3). Consequently, the biochemical

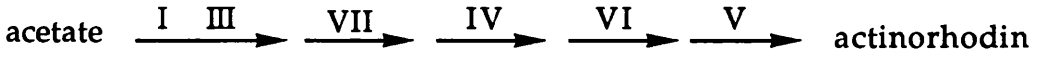
pathway was co-linear with the genetic order. Amino acid sequence comparisons revealed the organization of the functional domains to be similar to that of rat FAS, a type I FAS (Donadio and Katz, 1992). Therefore, the PKSs for polyacetate molecules appear to be type II while those for macrolides, and possibly polyethers, may be type I.

However, not all polyketides are synthesised via PKSs. Ashworth *et al.* (1988) showed that a polyketide intermediate of nonactin in *S, griseus* could be formed from dicarboxylic acids with condensation occurring to the α-position of one carboxylic group with loss of the other carboxylic group. In addition, use of $^{13}C$-labelled acetate and methyl groups resulted in labelling patterns in the polyketide backbone of brevetoxins which suggested the backbone was of mixed origins with possible involvement of dicarboxylic acids (Chou and Shimizu, 1987).

Polyketide antibiotics are produced from a large number of *Streptomyces*. Descriptions of the structure and formation of some of these antibiotics follow.

Actinorhodin is a chromosomally-determined, diffusible pigment excreted by *S. coelicolor* once growth has ceased (Wright and Hopwood, 1976a). It is red at neutral pH and blue in the alkaline conditions normally observed in non-pH-controlled cultures. Chemical analysis found actinorhodin to be a dimeric isochromanequinone, with the point of dimerization on C-10 (figure 1.4a), and each dimer was synthesized from eight acetate units via the polyketide pathway (Gorst-Allman *et al.*, 1981).

Co-synthesis experiments with seven non-producing mutants of *S. coelicolor* allowed a tentative proposal of the biosynthetic sequence of genes in the *act* pathway (Rudd and Hopwood, 1979):




(roman numerals denote mutant classes). An additional mutation, class II, was assumed to be regulatory (see section 1.7.2). Further characterization of the mutant classes was possible by the molecular cloning of all *act* genes (Malpartida and Hopwood, 1984), which revealed the genetic order to be:

(Malpartida and Hopwood, 1986). Class V mutants have since been split into two groups, $V_A$ and $V_B$ (Cole *et al.*, 1987), with subsequent studies revealing a sequence of:


(Malpartida *et al.*, 1987a).

Two intermediates of the actinorhodin biosynthetic pathway were isolated from class V mutants (Cole *et al.*, 1987). From these precursors of actinorhodin, Simpson (1991) suggested a chemical pathway from an unnamed yellow pigment to actinorhodin via kalafungin, which was added to the pathway proposed by Sherman *et al.* (1988) in figure 1.5.

Surprisingly, amino acid sequence analysis of *actIII* showed high similarity to human placental 17β-hydroxysteroid dehydrogenase and *Drosophila melanogaster* alcohol dehydrogenase (Baker, 1990). As described previously in this section, the *actIII* gene encodes a β-ketoreductase and, therefore, has functional similarity with eukaryotic dehydrogenases. Structural similarities also exist between actinorhodin and steroid signal molecules, and the antibacterial action of actinorhodin signifies intercellular communication, albeit with negative effects. Baker (1991) used these similarities to suggest that certain prokaryotic and eukaryotic dehydrogenases were derived from a common ancestor.

Related to actinorhodin is the polyketide antibiotic granaticin, produced by *S. violaceoruber* (Sherman *et al.*, 1989). Granaticin is also comprised of a benzoisochromanequinone moiety (figure 1.4b) and is synthesized from eight acetate units, but it contains a glucose molecule in the reduced form of 2,6-dideoxyhexose. The final step of the formation of granaticin is from dihydrogranaticin which suggests that the polyketide modification and sugar attachment stages take place prior to ring closure (Snipes *et al.*, 1979).

Other isochromanequinones include deoxyfrenolicin and frenolicin B (Tsuzuki *et al.*, 1986). They are nonaketides and have been isolated, in addition to their co-metabolites the octaketide nanaomycins, from *S. roseofulvus*. The co-production of these polyketides suggests the PKS of *S. roseofulvus* is able to utilize precursors of different chain lengths (Simpson, 1991).

Tetracyclines are polyketides made by condensation of malonyl-CoA units, which are formed by carboxylation of acetyl-CoA molecules (Thomas and Williams, 1983a; b; Wang and Vining, 1986). Their biosynthesis also requires two methionine residues. The antibiotic, tetracycline (figure 1.4c), is produced by *S. aureofaciens*, and resistance genes (*tetA* and *tetB*) have been found in *S. rimosus* and cloned into *S. griseus* (Ohnuki *et al.*, 1985a).

The entire gene cluster of oxytetracycline (figure 1.4d) has been cloned from *S. rimosus* and a biosynthetic pathway proposed (Binnie *et al.*, 1989) based on characterization of mutants blocked at various points in the pathway (Rhodes *et al.*, 1981).

Daunorubicin (figure 1.4e) is an anthracycline antibiotic produced by *Streptomyces* sp. C5 and *S. peucetius*. It is composed of a basic polyketide structure (ε-rhodomycinone, figure 1.4f) together with a partially deoxygenated hexose group, as in granaticin. Precursors for biosynthesis of daunorubicin were found to be propionyl-CoA, malonyl-CoA and thymidine 5'-diphosphate-D-glucose (TDPG; Bartel *et al.*, 1990b). The use of blocked mutants allowed elucidation of part of the biosynthetic pathway (Connors *et al.*, 1990a) and glycosylation was found to occur before methylation of the penultimate product (Connors *et al.*, 1990b).

Tetracenomycin C (figure 1.4g), another anthracycline, is produced by *S. glaucescens*. Mutational blocks in the biosynthetic pathway (Motamedi *et al.*, 1986) were used to propose a tentative sequence of events (Yue *et al.*, 1986). An *S. olivaceus* mutant blocked in tetracenomycin C production, *S. olivaceus* Tü 2353, produces tetracenomycin $B_3$. The blockage is at a branch point where the pathway diverts to either tetracenomycin C or ellaromycins (Simpson, 1991). The PKS genes for tetracenomycin C biosynthesis (*tcm* genes) were found to be similar to certain *act* genes: *orf1* and *orf2* encode a β-ketoacyl synthase, while the product of *orf3* is an ACP (Bibb *et al.*, 1989).

The similarity of PKS genes in several streptomycete species has allowed expression of these genes in heterologous hosts. In turn, this has led to the formation of hybrid polyketide structures, possibly with new antibacterial properties. A review on the formation of such novel polyketides is given by Strohl and Connors (1992). Additional, natural polyketide metabolites produced by *Streptomyces* and other organisms are also described in a recent review by Simpson (1991).

### 1.6.2 Macrolide antibiotics

Macrolides are related to polyketides in that they comprise a polyketide-derived macrocyclic ring but it is closed by a lactone bond. They also often contain one or more neutral or basic rare sugars (Martin and Liras, 1989a).

Erythromycins are composed of a 14-membered lactone ring (erythronolide) and the sugars desosamine and cladinose. There are four types of erythromycins: A, B, C and D. The major compound is erythromycin A (figure 1.4h; Seno and Hutchinson, 1986). Precursors of the polyketide structure were shown to be propionate and 2-methylmalonyl-CoA, which is formed by the carboxylation of propionyl-CoA (Cane *et al.*, 1986); the oxygen atoms are also derived from propionate (Cane *et al.*, 1981). It has been suggested that biosynthesis of erythromycins requires about 30 enzymatic steps (Vara *et al.*, 1989).

Several actinomycetes produce erythromycins, but the use of *Saccharopolyspora erythraea* biosynthetic and resistance genes as probes for different genera only revealed weak hybridization signals (Stanzak *et al.*, 1990). It was suggested that the genes may have been transferred horizontally between genera early in the stages of actinomycete divergence. However, lack of data did not allow comparison with other erythromycin PKSs and, therefore, determination of the extent of genetic evolution within them.

Tylosin is a 16-membered macrolide containing mycaminose, mycarose and mycinose (figure 1.4i), and is produced by *S. fradiae* (Seno and Hutchinson, 1986). The initial lactone precursor is tylactone which is formed from five propionate molecules, two acetates and a butyrate molecule (Omura *et al.*, 1975). Rate limiting steps in tylactone biosynthesis were proposed to be the construction of the CoA derivatives of these substrates (Omura *et al.*, 1977). The amino acid origin of the precursors was elucidated by Dotzlaf *et al.* (1984) using $^{14}$C-labelled residues and related compounds. They found that methionine and isoleucine, in addition to propionate, provided propionyl CoA, while leucine and phenylalanine were used for butyryl CoA and ethylmalonyl CoA formation. It was also discovered that isobutyrate, an intermediate in valine catabolism, was converted to the propionate and butyrate units used in tylosin biosynthesis (Omura *et al.*, 1983).

Mutants blocked in tylosin production were classified into nine groups, *tylA*

to *tylI* (Baltz and Seno, 1981), and a biosynthetic pathway from tylactone to tylosin was proposed (Cox *et al.*, 1986).

Other macrolides, the avermectins are produced by *S. avermitilis* (Albers-Schönberg *et al.*, 1981; Burg *et al.*, 1979; Egerton *et al.*, 1979), and are oleandose disaccharide derivatives of 16-membered pentacycline lactones (Schulman, 1989). They are synthesized from seven acetates, five propionates, and a 2-methylbutyrate (from isoleucine) or isobutyrate (from valine; Cane *et al.*, 1983). Deamination and decarboxylation of isoleucine (or valine) occurs first in the pathway, providing a primer for addition of the acetates and propionates via polyketide synthesis steps. The oleandose groups originate from glucose and are methylated prior to attachment to the macrolide ring (Schulman, 1989)

### 1.6.3 β-lactam antibiotics

Several actinomycete genera, including *Streptomyces*, produce β-lactam antibiotics such as cephalosporins and cephamycins. The β-lactam-thiazolidine ring is derived from linked L-α-aminoadipic acid, L-cysteine and L-valine (the tripeptide LLD-ACV). Cyclization occurs via the catalytic activity of isopenicillin N synthase resulting in isopenicillin N. Penicillin is then formed from isopenicillin N in fungi by exchange of the α-aminoadipyl group for phenylacetic acid (Martin and Liras, 1989a). Cephamycin (figure 1.4j) is produced by *S. lactamdurans* and *S. clavuligerus*: isopenicillin N is converted to cephamycin C via a biosynthetic pathway incorporating penicillin N, deacetoxycephalosporin C, deacetylcephalosporin C and cephalosporin C (Martin and Liras, 1989a). Cloning of β-lactam biosynthetic genes has revealed similarities in the isopenicillin N synthase genes of fungi, *S. clavuligerus* (Leskiw *et al.*, 1988) and *S. lipmanii* (Weigel *et al.*, 1988). It has been suggested the β-lactam genes evolved in *Streptomyces* and were horizontally transferrred to a fungal progenitor 370 million years ago (Weigel *et al.*, 1988).

The deacetoxycephalosporin C synthase and hydroxylase activities have been found in a single polypeptide from *Aspergillus chrysogenum* (Samson *et al.*, 1987), whereas, in S. *clavuligerus*, each function has been detected on a separate polypeptide (Kovacevic *et al.*, 1989).This organization of enzymic activities is analogous to that of PKSs in fungi and *Streptomyces* (section 1.6.1).

### 1.6.4 Aminoglycosides

Aminoglycoside antibiotics include streptomycin (Martin and Liras, 1989a), neomycin (Majumdar and Majumdar, 1970) and vancomycin (Mertz and Doolin, 1973), among others. Streptomycin (*N*-methyl-α-L-glucosamine-(1''-2')-α-L-streptose-(1'-4)streptidine; figure 1.4k) is produced by *S. griseus* and is formed from *myo*-inositol via a biosynthetic pathway incorporating glutamine, arginine, alanine (or glutamine), deoxy-TDP-glucose and NDP-*N*-methyl-L-glucosamine (Ohnuki *et al.*, 1985b).

### 1.6.5 Other antibiotics

These are antibiotics which do not belong to specific groups.

Undecylprodigiosin (red; figure 1.4l) is a non-polar, non-diffusible pigment secreted by *S. coelicolor* (Tsao *et al.*, 1985). It is structurally related to prodigiosin, a red pigment produced by *Serratia marcescens* (Williams, 1973), but contains a longer polyketide chain (Wasserman *et al.*, 1976); biosynthesis of undecylprodigiosin is therefore assumed to be similar to prodigiosin. Prodigiosin contains three pyrrole rings which are formed separately as a bipyrrole and monopyrrole. The bipyrrole was found to be synthesised from proline, serine, acetate and the methyl group of methionine, while the monopyrrole was formed from alanine and polyacetate (Wasserman *et al.*, 1973). In actinomycetes, the monopyrrole is partly assembled as a polyketide chain (Gerber *et al.*, 1978); undecylprodigiosin could, therefore, also be classed as a polyketide. Mutants of *S. coelicolor* unable to transport proline overproduced undecylprodigiosin, indicating the amino acid's involvement in secondary metabolism and the possibility that proline biosynthesis was not feedback regulated (Hodgson *et al.*, 1990).

The undecylprodigiosin biosynthetic genes are located, like actinorhodin, as a single cluster on the *S. coelicolor* chromosome (Rudd and Hopwood, 1980). Early co-synthesis experiments with mutants blocked in undecylprodigiosin production revealed five mutant classes (*redA* to *redE*; Feitelson and Hopwood, 1983), which were all possibly blocked in the bipyrrole branch of the pathway (Tsao *et al.*, 1985). Further studies have shown that a minimum of 18 genes are required for undecylprodigiosin biosynthesis, with possible coordinate expression of genes in both pathway

arms (Feitelson et al., 1985; 1986; Coco *et al.*, 1991). This suggests regulation of the pathway is complex.

A third antibiotic produced by *S. coelicolor* is methylenomycin (Wright and Hopwood, 1976b). However, unlike the two previously mentioned, the biosynthetic gene cluster for methylenomycin (*mmy*) is present on the naturally occurring plasmid, SCP1 (Chater and Bruton, 1985; Kirby *et al.*, 1975). The antibiotic is an epoxycyclopentane (2-methylene-cyclopentan-3-one-4, 5-epoxy-4, 5-dimethyl-1-carboxylic acid; figure 1.4m), but the biosynthetic pathway is not well characterized (Martin and Liras, 1989a). However, co-synthesis tests had resulted in five classes of mutants (Kirby and Hopwood, 1977).

Other types of secondary metabolites include those containing amino acid residues. Bialaphos (figure 1.4n), produced by *S. hygroscopicus* and *S. viridochromogenes*, is a tripeptide herbicide, composed of two L-alanine residues and an L-glutamate analogue, phosphinothricin (Seto *et al.*, 1982). It is synthesized from pyruvate or phospho*enol*pyruvate in at least thirteen steps (Hara *et al.*, 1988; Murakami *et al.*, 1986; Thompson and Anzai, 1987). On the other hand, actinomycins, synthesized by *S. antibioticus*, contain pentapeptides. They are chromopeptide antibiotics with two pentapeptides attached to a phenoxazinone chromophore, actinocin (Martin and Liras, 1989a). Precursors of biosynthesis are D-fructose and L-glutamate which contribute carbon atoms to the sarcosine and methylvaline, and proline and L-threonine respectively (Inbar and Lapidot, 1988). The final stage in the biosynthetic pathway of actinomycin D (figure 1.4o) is condensation of two molecules of 4-methylhydroxylanthraniloyl pentapeptide (Jones and Hopwood, 1984).

The final antibiotic to be mentioned in this section is chloramphenicol (figure 1.4p). It is unique in that it is not synthesized from a final product in a primary pathway of *S. venezuelae*, but it branches from chorismic acid, an intermediate in the shikimate pathway (Vining and Westlake, 1984). Analysis of blocked mutants revealed a pathway which involved six sequential steps and one parallel step from chorismic acid, including the addition of a dichloroacetyl derivative (Doull *et al.*, 1985).

## 1.7 Catabolite regulation of secondary metabolite production

Internal and external regulation of differentiation and secondary metabolism in *Streptomyces* have been extensively reviewed and updated over the years (Martin and Demain, 1980; Martin and Liras, 1989a; Shapiro (ed.), 1988). It is important here, however, to mention some aspects of regulation in *Streptomyces*.

### 1.7.1 Catabolite repression, and regulation by oxygen

Secondary metabolism is mainly associated with a decrease in growth rate or cessation of growth in vegetative mycelia. It is evident, therefore, that nutritional availability affects secondary metabolite production.

Glucose is the favoured carbon source (C-source) for growth of *Streptomyces*. Uptake and utilization of glucose proceeds prior to that of a C-source effecting slower growth. In combination with glucose, a second C-source is mainly used for secondary metabolite biosynthesis (Martin and Demain, 1980; Demain, 1988). Glucose repression (carbon catabolite repression) of a number of streptomycete antibiotic synthetase genes has been reported (Demain, 1988). For example, the most well studied antibiotic biosynthetic pathway subject to carbon catabolite repression is that of actinomycin. Four genes involved in the formation of actinomycin exhibit glucose repression: phenoxazinone synthase (Gallo and Katz, 1972), hydroxykynureninase, kynurenin formamidase II and tryptophan pyrrolase (Katz, 1980). Two of the cephalosporin biosynthetic enzymes are also glucose repressible: δ–[α-aminoadipyl]cysteinylvaline synthetase and deacetoxy-cephalosporin C synthetase (Cortes *et al.*, 1986). Fungal β-lactam producers, however, exhibit glucose repression of the isopenicillin N gene (*ipnA*) but not of the δ–[α-aminoadipyl]cysteinylvaline synthetase gene (*acvA*) (Brakhage *et al.*, 1992; Espeso and Penalva, 1992). Outright carbon catabolite repression was not observed to affect arylamine synthetase during chloramphenicol production by *S. venezualae*, but enzymic activity was greatly reduced in the presence of excess glucose when the idiophase was associated with nitrogen depletion (Bhatnagar *et al.*, 1988). Nevertheless, timing of synthesis of the biosynthetic enzyme remained unaltered.

Cyclic adenosinemonophosphate (cAMP) has been shown to reverse the effects of carbon catabolite repression in *E. coli* (Magasanik and Neidhardt,

1987). Although a few reports have stressed the involvement of cAMP in the reversal of carbon repression of antibiotic biosynthesis in *Streptomyces*, the majority of evidence seems to indicate that the nucleotide does not have a role similar to that in the enteric bacteria (Demain, 1988). Nevertheless, cAMP may respond to physiological changes in *Streptomyces*. An increase in concentration of cAMP was observed prior to entry of *S. griseus* into stationary phase. This was followed by a sharp decrease in concentration (90%) which occurred simultaneously with early exhaustion of phopshate. Levels of cAMP then remained low throughout streptomycin production (Ragan and Vining, 1978).

Another form of catabolite repression is by nitrogen. The regulatory effects on secondary metabolite production by nitrogen are comprehensively reviewed by Shapiro (1988). The type of nitrogenous compound used in growth media is important: ammonium is readily utilizable and promotes high growth rates, but it represses the synthesis of many antibiotics including tylosin (Tanaka *et al.*, 1986), erythromycin (Flores and Sanchez, 1985), streptomycin (Woodruff and Ruger, 1948) and cephamycin C (Brana *et al.*, 1986a; Castro *et al.*, 1985). Nitrogen sources supporting low growth rates are the most suitable for antibiotic production (Khaoua *et al.*, 1991).

It was thought that the nitrogen assimilation enzyme, glutamine synthetase, was involved in nitrogen regulation of cephalosporin production by *S. clavuligerus* (Castro *et al.*, 1985). However, no correlation has been found between glutamine synthetase activities and those of the cephalosporin enzymes, β-lactam cyclase and expandase, in different media (Brana *et al.*, 1986b). Nevertheless, the suggestion that valine dehydrogenase was subject to nitrogen repression and was involved in regulation of tylosin biosynthesis by *S. fradiae* (Omura *et al.*, 1983) has been supported by studies on the enzyme in *S. coelicolor* (Navarette *et al.*, 1990). The gene encoding threonine dehydratase may also be a site of nitrogen catabolite repression in *S. fradiae* (Lee and Lee, 1991).

Several antibiotic groups exhibit sensitivity to phosphate levels during their biosynthesis (Aharonowitz and Demain, 1977). However, phosphate regulation of the β-lactams requires much higher phosphate concentrations, e.g., cephalosporin biosynthesis is decreased in the presence of 100mM phosphate (Lübbe *et al.*, 1985). Several aminoglycosides require phosphatases for formation of the active product, but phosphatase genes are repressed by high phosphate levels and the enzymes may be inhibited. For

example, streptomycin-6-phosphate accumulated during synthesis of streptomycin in the presence of high phosphate concentrations (Miller and Walker, 1971), and the phosphatases involved in the dephosphorylation of neomycin and vancomycin precursors were possibly both genetically repressed and inhibited by phosphate (Majumdar and Majumdar, 1970; Mertz and Doolin, 1973). Other antibiotic biosynthetic enzymes whose synthases are under phosphate control include, anhydrotetracycline oxygenase (Behal *et al.*, 1979), *p*-aminobenzoic acid (PABA) synthase, which is involved in the synthesis of aromatic polyene macrolides (Gil *et al.*, 1985), tylosin enzymes (Madry and Pape, 1982) and both cephamycin synthetase and clavulanic acid synthetase of *S. clavuligerus* (Lebrihi and Lefebvre, 1987).

Cyclic AMP, adenosine triphosphate (ATP), guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) have all been suggested to be involved in mediating phosphate control (Martin, 1988). Strong evidence is not yet available to state whether these molecules do have a role although changes observed in the ATP pool during growth of *S. griseus* may indicate an association of the nucleotide with candicidin production (Liras *et al.*, 1977). Increased levels of ppGpp and pppGpp at the onset of antibiotic production may only be indicative of a normal stringent response (section 1.5.1).

Different nutrient concentrations in media permit different patterns of antibiotic production, *e.g.*, antibiotic production exhibiting nitrogen or phosphate regulation may become dependent on the growth rate under certain conditions (see Rhodes, 1984). Biphasic fermentations of *S. coelicolor* have been obtained by manipulating phosphate and nitrogen levels, to which actinorhodin is sensitive (Doull and Vining, 1990; Hobbs *et al.*, 1990); distinct phases of growth and actinorhodin production were achieved (Doull and Vining, 1989). In addition physiological steering obtained exclusive synthesis of methylenomycin by *S. coelicolor* (Hobbs *et al.*, 1992) while cephamycin production was separated from clavulanic acid production by *S. clavuligerus* by adjusting the phosphate level (Romero *et al.*, 1984).

*Streptomyces* are obligate aerobes and, therefore, oxygen concentrations within cultures will have a bearing both on growth and on antibiotic production. Oxygen limitations can occur in streptomycete fermentations because of the viscous nature of hyphal growth (Steel and Maxon, 1966) and,

therefore, affect productivity. For example, a reduction in avermectin production by *S. avermitilis* was found at dissolved oxygen concentrations (DO) of below 20% (Buckland *et al.*, 1985) while levels of cephalosporin in cultures of *S. clavuligerus* fell by a factor of three at a DO of less than 75%, when oxygen in the inlet gas was reduced to 14% (Yengneswaran *et al.*, 1988). However, in contrast to cephalosporin production, cephamycin C production by *S. clavuligerus* was increased by two to three fold at DO levels of 50% to 100%. This showed the difference in effect of DO levels on different antibiotics. Maximum productivity in penicillin fermentations were shown to require the maintenance of DO levels above a minimum value (Vardar and Lilly, 1982).

High stirrer rates can open networks of mycelia thus improving mixing and nutrient (including oxygen) mass transfer. This was observed in *S. niveus* cultures where the critical DO was reduced (Wang and Fewkes, 1977). A high stirrer speed (990rpm, 1300rpm) maintained DO levels at greater than 90% air saturation in *S. clavuligerus* fermentations (Belmar-Beiny and Thomas, 1991). At low speeds (490rpm), however, DO levels fell to 60%.

Production of additional metabolites may occur at high DO levels. Macrocin, the final intermediate in tylosin biosynthesis, was produced together with tylosin when *S. fradiae* was cultivated at controlled high DO levels (> 90% saturation) and high aeration rates ($1v.v^{-1}.min^{-1}$; Chen and Wilde, 1991). Production of macrocin stopped after a reduction in either aeration rate or DO level. It is thought that a high DO level may have increased the activities of enzymes preceding macrocin formation and, since conversion to tylosin may have been limited by an *O*-methylation step (Seno and Baltz, 1982), accumulation, and therefore secretion, of macrocin occurred.

Increasing the oxygen partial pressure ($P_{O_2}$) resulted in increased specific formation of tetracycline by *S. aureofaciens* and *S. rimosus*, although biomass formation was decreased (Liefke *et al.*, 1990). Oxygenation of the tetracycline molecule also occurred at high $P_{O_2}$, presumably because of increased activity of the oxygenase involved in oxytetracycline biosynthesis. Improved oxygen utilization by the producing organism can also increase antibiotic production, as shown by a ten-fold increase in actinorhodin production by *S. coelicolor* expressing *Vitreoscilla* haemoglobin (Magnolo *et al.*, 1991).

### 1.7.2 Streptomycete regulatory factors and pleiotropic effectors

Many factors in *Streptomyces* have been found to be involved in regulation of differentiation or secondary metabolism or both. Factors affecting both processes are termed pleiotropic effectors (Hopwood, 1988).

Differentiation mutants of *S. coelicolor*, lacking the ability to form aerial mycelia and therefore not achieving a white powdery appearance, are described as *bld* (bald) mutants (Chater and Hopwood, 1973). *bld* mutants are also defective in the production of secondary metabolites (Chater, 1989). The developmental block in mutant classes, *bldA*, *bldG* and *bldH*, may be relieved by growth on carbon sources other than glucose, although antibiotic biosynthesis was only restored in *bldH* mutants (Champness, 1988). It was suggested there may be alternative means of regulation of differentiation and secondary metabolism, one of which is glucose repressible. Development of aerial mycelium by wild type *S. alboniger*, *S. scabies* and *S. coelicolor* had previously been shown to be glucose repressible, which was not reversed by addition of cAMP (Redshaw *et al.*, 1976).

The *bldB* region has recently been shown to contain two genes (*bldB* and *bldI*), both of which are involved in morphological development although their gene products have not been identified (Harasym *et al.*, 1990). However, the *bldA* gene product has been identified as a tRNA required for translation of an extremely rare leucine codon, TTA, in *S. coelicolor* at the end of growth phase (Lawlor *et al.*, 1987). Expression of genes containing a TTA codon is abolished in *bldA* mutants (Leskiw *et al.*, 1991). *bldA* mutants are lacking in the ability to produce undecylprodigiosin but *red* biosynthetic genes do not appear to contain TTA codons, suggesting that elimination of the tRNA is not directly responsible for lack of the antibiotic (Guthrie and Chater, 1990). Instead, the $tRNA^{Leu}$ may be required for translation of effector molecules involved in regulation of secondary metabolism and differentiation (Chater, 1990). This is supported by observed production of actinorhodin in *bldA* mutants containing additional copies of the *actII* regulatory gene (Passantino *et al.*, 1991; this section).

Other *S. coelicolor* mutants defective in differentiation are *whi* (white) mutants. They form aerial mycelia but are unable to sporulate and so colonies remain white (Hopwood *et al.*, 1970). Possible functions of *whi* gene products are given by Chater and Merrick (1979), but it is known that

they are involved in initial stages of sporulation (Chater, 1972). The *whiG* gene of *S. coelicolor* has been cloned (Mendez and Chater, 1987) and subsequent sequence determination has shown that it encodes a sigma ($\sigma$) factor similar to the motility factor ($\sigma^{28}$) of *Bacillus subtilis* (Chater *et al.*, 1989). $\sigma$ factors are subunits required to form the active form (holoenzyme) of RNA polymerase, the enzyme responsible for gene transcription (as reviewed by Helmann and Chamberlin, 1988). Over-expression of *whiG* in vegetative hyphae of *S. coelicolor*, resulted in sporulation, thus the presence and level of WhiG protein is important in the developmental fate of hyphae (Mendez and Chater, 1987).

Differential expression of genes by alternative $\sigma$ factors with different promoter specificities is well known in *B. subtilis* (see Karmazyn-Campelli *et al.*, 1989) as is the occurrence of several forms of RNA polymerase holoenzyme in *S. coelicolor* A3(2) (Buttner, 1989). However, excepting *whiG*, the roles of other $\sigma$ factors in *S. coelicolor* development have not yet been discovered (Buttner *et al.*, 1990).

Differentiation and secondary metabolism in *Streptomyces* are also controlled by autoregulatory factors produced by the organisms. For example, *S. griseus* requires the presence of A-factor (2-*S* isocapryloyl-3-*S*-hydroxymethyl-$\gamma$-butyrolactone; figure 1.4q), at very low concentrations (Hara and Beppu, 1982) for sporulation and streptomycin production (a good review on A-factor is given by Gräfe, 1988). *S. griseus* also secretes L-factor which may have a similar structure to A-factor; other A-factor-like pheromones (communication signals) are produced by several actinomycete species (Stephens, 1986). Virginiamycin production by *S. virginiae* is dependent on the presence of three butanolides: virginiae butanolides (VB) A, B and C (Yamada *et al.*, 1987). Specific binding proteins for A-factor (Miyake *et al.*, 1989) and for VB C (Kim *et al.*, 1989) have been isolated and suggested to mediate the pleiotropic signal imparted by the pheromones. A-factor binding protein may repress genes involved in sporulation and streptomycin production, derepression occurring when A-factor binds to the binding protein (Miyake *et al.*, 1990).

*S. coelicolor* also synthesises A-factor but it is not required either for sporulation or antibiotic production (Hara *et al.*, 1983). Horinouchi *et al.* (1983; Horinouchi and Beppu, 1984) isolated a gene, *afsB*, from *S. coelicolor* which stimulated synthesis of A-factor in strains of *S. lividans* deficient in A-factor production. It was also responsible for effecting synthesis of

actinorhodin and undecylprodigiosin in this organism under conditions in which they are not normally produced. Sequence analysis of *afsB* indicated the gene may encode a DNA-binding protein (Horinouchi *et al.*, 1986) and subsequent introduction of the gene into an *afs*B$^-$ mutant of *S. coelicolor* (which did not produce actinorhodin, undecylprodigiosin or A-factor) restored transcription of *act* genes (Horinouchi *et al.*, 1989). Recently, however, Stein and Cohen (1989) cloned a separate chromosomal locus from *S. lividans*, *afsR*, which complemented the *afsB*$^-$ phenotype by restoring pigment production; *afsB* was therefore renamed *afsR*. Purification of the AfsR protein showed that both N-terminal and C-terminal halves could effect slight antibiotic production from *S. lividans* (Horinouchi *et al.*, 1990) and that the protein could be phosphorylated by a phosphokinase activity present in both *S. coelicolor* and *S. lividans* (Hong *et al.*, 1991).

Mutations in additional loci on the *S. coelicolor* chromosome, *absA* (Adamidis *et al.*, 1990) and *absB* (Champness *et al.*, 1990) resulted in cessation of production of the full complement of *S. coelicolor* antibiotics, actinorhodin, undecylprodigiosin, methylenomycin and calcium-dependent antibiotic (CDA, a chromosomally-determined, channel-forming antibiotic; Lakey *et al*, 1983; Hopwood and Wright, 1983). However, mutations in *absA* do not affect sporulation (Adamidis *et al.*, 1990). *absA* and *absB* gene products may therefore be involved in a regulatory mechanism common to all the antibiotics. A locus which may act closer in the mechanism to the antibiotic gene clusters is *abaA* (Fernandez-Moreno *et al.*, 1992). Mutation of this locus reduced or abolished production of three antibiotics but not methylenomycin. Regulation by these genes, therefore, seems to be via a complex network of controls.

Proteinaceous factors have also been implicated in the regulation of differentiation. For example, *S. griseus* secretes factor C which induces aerial hyphae formation and sporulation and has been immunologically detected in 23 stretomycete species and in *B. subtilis* (Szeszak *et al.*, 1985). In addition, suggestions have been made that SapB, a small sporulation protein from *S. coelicolor*, acts as a pheromone for the formation of aerial hyphae, which it restored in a recently found *bld* mutant (Willey *et al.*, 1991).

An additional molecule thought to be involved in regulation of both differentiation and secondary metabolism in *Streptomyces* is ppGpp. *Streptomyces* exhibit a stringent response similar to enteric bacteria under

conditions of amino acid limitation (section 1.5.1), possibly mediated by ppGpp (Riesenberg *et al.*, 1984). Mutants of *Streptomyces* sp. MA406-A-1 reduced in the ability to accumulate ppGpp during amino acid limitation (relaxed, *rel*, mutants) did not produce the secondary metabolite, formycin (Ochi, 1986a). This suggested that the stringent response of ppGpp accumulation was related to formycin production. The *rel* mutants also exhibited a delay in aerial mycelium formation possibly due to a less significant decrease in the intracellular GTP pool. ppGpp inhibits IMP dehydrogenase and therefore GTP synthesis (Cashel and Rudd, 1987) and a decrease in GTP content had been shown to correlate with differentiation (Ochi, 1986b).

*rel* mutants of *S. griseus*, *S. antibioticus* and *S. coelicolor* showed a similar failure to produce streptomycin (Ochi, 1987a), actinomycin (Ochi 1987b) and A-factor and undecylprodigiosin (Ochi, 1990) respectively. Aerial mycelium formation was also delayed in all three mutant strains. Subsequent molecular studies indicated ppGpp may also control expression of genes involved in actinomycin biosynthesis (Kelly *et al.*, 1991). However, actinorhodin production by the *S. coelicolor rel* mutant was not completely prevented and appeared after 10 days incubation (Ochi, 1990). Strauch *et al.* (1991) isolated a *rel* mutant of *S. coelicolor*, resembling that examined by Ochi (1990), which was observed to produce both actinorhodin and undecylprodigiosin. Suppression of ppGpp accumulation by the parent strain did not hinder antibiotic production. Therefore, it was suggested that a transient increase in ppGpp was "required, but not sufficient" for initiation of secondary metabolism in *S. coelicolor*. The stringent response has also been shown not to be associated with antibiotic production in *S. clavuligerus* (Bascaran *et al.*, 1991), and ppGpp levels were found to fall prior to streptomycin production in *S. griseus* (An and Vining, 1978).

Genes implicated in regulation of antibiotic production have been located within antibiotic gene clusters. *actII*, a gene in the actinorhodin cluster, was suggested to be regulatory because *actII* mutants failed to produce any intermediates or actinorhodin in co-synthesis experiments with other *act* mutants (Rudd and Hopwood, 1979; Malpartida and Hopwood, 1986, Fernandez-Moreno *et al.*, 1991). Sequence analysis of *actII* revealed four ORFs, two of which contained TTA codons and were therefore *bldA*-dependent (this section): *orf2*, encoding a putative transmembrane export protein, and *orf4*. The product of *orf4* is a transcriptional activator gene for actinorhodin structural genes and has been shown to be similar to the *redD*

gene (Fernandez-Moreno *et al.*,1991). *redD* mutants do not co-synthesise undecylprodigiosin (Rudd and Hopwood, 1980) allowing the assumption that the gene product is involved in transcription of other *red* genes (Malpartida et al., 1990). Cloning of the *redCD* region into *S. coelicolor* resulted in overproduction of the red pigment (Narva and Feitelson, 1990).

Introduction of extra copies of genes present in the daunorubicin biosynthetic cluster, $dnrR_1$ and $dnrR_2$, into *S. peucetius* caused overproduction of the antibiotic (Otten *et al.*, 1990). Stutzman-Engwall *et al.* (1992) showed that the predicted amino acid sequence of *dnrI*, one of two ORFs in $dnrR_1$, was highly similar to *actII-orf4* and *redD*. The genes were therefore classed together in one family which also included *afsR* (which is described previously in this section). Amino acid sequences of *afsR* and *redD* included helix-turn-helix motifs, indicative of DNA binding proteins. A second family of putative regulatory genes seemed to encode protein kinases or other proteins involved in a two-component regulatory system. Their amino acid sequences also contained helix-turn-helix motifs. This family included the second ORF in $dnrR_1$, *dnrJ*, the *Saccharopolyspora erythraea eryC1*, a regulatory gene in erythromycin production (Dhillon *et al.*, 1989), *strS*, a putative regulatory gene involved in streptomycin biosynthesis by *S. griseus* (as cited by Stutzman-Engwall *et al.*, 1992), and the *S. hygroscopicus brpA*, which regulates transcription of bialaphos biosynthetic and resistance genes (Anzai *et al.*,1987; Raibaud *et al.*, 1991). 27 gene products are dependent on *brpA* for optimal expression (Holt *et al.*, 1992).

Additional regulatory genes not previously mentioned are the *S. griseus strR*, which, like *strS*, is involved in streptomycin regulation (Ohnuki *et al.*, 1985b; Distler *et al.*, 1987), and one implicated in regulation of methylenomycin production (Chater and Bruton, 1985). In contrast to other reported regulatory genes, the methylenomycin (*mmy*) gene seems to act through negative regulation.

## 1.8 Primary metabolism in *Streptomyces*

### 1.8.1 Introduction

Primary metabolic processes in bacteria are those required for growth and reproduction. Any good Biochemistry or Microbiology text book will

contain detailed descriptions of these processes, but it is pertinent here to give a short overview. Elucidation of major pathways of primary metabolism was achieved using *E. coli*, therefore all references are to pathways present in this bacterium.

Breakdown of carbon and energy sources take place via catabolic pathways which converge onto the central metabolic pathways, mainly at pyruvate. The central metabolic pathways (CMPs) include the Embden-Myerhof-Parnas pathway (EMP, also known as glycolysis), the pentose phosphate pathway (PP, or hexosemonophosphate shunt), and the citric acid cycle (or tricarboxylic acid cycle, TCA). The TCA cycle is the final oxidation pathway common to carbon substrates such as carbohydrates, amino acids and fatty acids. Growth on fatty acids, however, induces the enzymes of the glyoxylate bypass. This is necessary to maintain concentrations of certain intermediates in the TCA cycle, which are used as precursors and therefore continually drained from the cycle. During growth on carbohydrates, perpetuation of the cycle occurs due to anaplerotic pathways stemming from the EMP pathway.

Oxidation of carbon substrates in aerobic chemoheterotrophs occurs concomitantly with reduction of electron acceptors or carriers. These include the molecules nicotinamide adenine dinucleotide (NAD), flavinoid adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADP) and Coenzyme-A (CoA). The terminal electron acceptor is $O_2$ to which electrons are transferred from NAD or FAD via a specifically orientated electron transport chain containing cytochromes. As electrons flow along the chain, adenine triphosphate (ATP), a free energy donor, is formed from ADP and inorganic phosphate ($P_i$). According to Mitchell's chemiosmotic hypothesis (Mitchell, 1961), electron transport and ATP synthesis are coupled by a proton gradient caused by the translocation of protons from the chain through the inner membrane. The proton-motive force generated because of the inability of protons to return to the cytoplasm except through specific sites, is postulated to drive the synthesis of ATP by an anisotropic ATPase in the inner membrane. This process is known as oxidative phosphorylation. However, ATP synthesis also occurs during glycolysis by substrate-level phosphorylation.

Energy provided by hydrolysis of ATP is used for the biosynthesis of cellular material and also for some uptake mechanisms. Reducing power is also required for the formation of some small molecules and this is provided by NADPH (the reduced form of NADP). Biosynthesis is also termed

anabolism and, as mentioned previously, precursors used in biosynthetic pathways are intermediates in catabolic pathways. Although there are over 20 different amino acids, several nucleotides and fatty acids, many are products of common biosynthetic pathways and all share only a few precursors (see section 1.10). For example, amino acids may be grouped into six families (figure 1.6). Early experiments to elucidate amino acid precursors in *E. coli* were carried out by Roberts *et al.* (1955) using radiolabelled substrates. Catabolism and anabolism are therefore interdependent and together comprise primary metabolism.

### 1.8.2 Streptomycete primary metabolism

Early studies on primary metabolism in *Streptomyces* concentrated on ascertaining glucose utilization pathways using radiolabelled glucose and intermediates from the central pathways as substrates. The use of radiolabelled glucose gave an approximation of the relative utilization of these pathways (Wang *et al.*, 1958a): during glycolysis carbons 3 and 4 from glucose are released as $CO_2$, because of the formation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Use of the PP pathway, however, results in release of C-1 as $CO_2$. The extent of incorporation of $^{14}C$ into $CO_2$ therefore indicated which pathway was in use. Early studies on *S. coelicolor* and *S. reticuli* suggested initial stages of glucose metabolism may have been via the PP pathway (Cochrane *et al.*, 1953): oxidation of suitable substrates also revealed the presence of some EMP pathway enzymes. However, later studies identified the EMP pathway to be a major pathway of glucose metabolism in *S. griseus*. The PP pathway played a minor role, but this was dependent on the age of the mycelium. Older mycelia exhibited greater use of this pathway but the overall pattern of pathway utilization was the same as that in younger mycelia (Wang *et al.*, 1958b; Cochrane, 1961). The presence of all the glycolytic enzymes had been determined in *S. coelicolor* prior to this (Cochrane, 1955). Later experiments revealed the presence of the EMP enzymes, phosphofructokinase and pyruvate kinase, in *S. alboniger* (Surowitz and Pfister, 1985a), and phosphofructokinase and fructose-1,6-bisaldolase in *Streptomyces* C5, *S. lividans* and *S. aureofaciens* (Dekleva and Strohl, 1988a). Activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, enzymes of the PP pathway, were also detected in the latter study.

The existence of TCA enzymes in *Streptomyces* was also being determined

during the 1950's: cell-free extracts of *S. coelicolor* were shown to oxidize TCA cycle intermediates in an order corresponding to that of the cycle (Cochrane and Peck, 1953). In addition, non-proliferating cells of *S. griseus* accumulated glutamate that had incorporated radioactivity from $^{14}$C-acetate into the α- and γ-carboxylic groups. The proportion of incorporation was indicative of successive turns of the TCA cycle via asymmetrical distribution of carbon into oxaloacetate (Gilmour *et al.*, 1955). The study also verified that intermediates from the TCA cycle were used as precursors in the biosynthetic pathways, and were thought to be provided by the use of anaplerotic pathways. Uptake of $^{14}CO_2$ by intact cells of *S. griseus*, in the presence of pyruvate, resulted in high incorporation into aspartate and glutamate with preferential labelling of the β-COOH of aspartate (Butterworth *et al.*, 1955). It was suggested that carboxylation of pyruvate occurred to form malate or oxaloacetate. However, Cochrane (1955) had not detected pyruvate carboxylase activity in *S. coelicolor*. Subsequent interest in anaplerotic pathways in *Streptomyces* has resulted in the purification of phospho*enol*pyruvate carboxylase (PEPC) from *S. coelicolor* (H. Bramwell, manuscript in preparation). Activity of this enzyme had been proposed previously to be responsible for incorporation of $^{14}$C from $CO_2$ into succinate by *S. coelicolor* (Cochrane, 1952). PEPC from *Streptomyces* C5 has also been partly purified (Dekleva and Strohl, 1988b).

Enzymes of a glyoxylate bypass, or similar, were shown to be present in *S. griseus* when precursors for biomass and streptomycin production were produced during growth on oils (Perlman and Wagman, 1952). However, no glyoxylate bypass activity was detected in Streptomyces C5 (Dekleva and Strohl, 1988a), which, like *S. peucetius* (Dekleva *et al.*, 1985) grew poorly on yeast extract medium containing acetate in place of glucose.

Although the experiments carried out by Cochrane and co-workers established the presence of some enzymes of the central metabolic pathways, since then very little work has been carried out to purify and characterize these enzymes. Nevertheless, recent studies have been performed with the aim of identifying genetic and metabolic switches between primary and secondary metabolism. Since secondary metabolites use intermediates or biosynthetic products from primary metabolism as precursors (section 1.6), the flow of large quantities of these precursors to secondary metabolism must be achieved by altering the regulation of activities of central pathway enzymes. Only by acquiring sufficient information about the organization and regulation of primary metabolic genes to match that of the genes

involved in differentiation and secondary metabolism, will an understanding of the switches be acquired (Hopwood *et al.*, 1986). In addition, an overall description of the streptomycete life cycle, thus obtained, would allow their full genetic potential to be realized (Hunter, 1988). Most of the present studies on primary metabolism have, therefore, been carried out in relation to antibiotic production with very few, unfortunately, concentrating solely on primary metabolism in its own right.

Since anaplerotic reactions are important for the provision of precursors to antibiotic biosynthesis, enzymes involved in these pathways have been purified from *S. coelicolor* and their genes cloned. PEPC, for example was found to be very similar to other prokaryotic PEP carboxylases (H. Bramwell, manuscript in preparation). *Iso*citrate dehydrogenase (ICDH), which has a main role in regulation of the glyoxylate bypass, has been shown, however, to be much larger than that of *E. coli* and, unlike the *E. coli* enzyme, does not undergo covalent modification by phosphorylation (Taylor, 1992). Isocitrate lyase (ICL) activity has also been detected in *S. coelicolor* (P. Chapman, personal communication), contrary to the findings of Dekleva and Strohl (1988a) with *Streptomyces* C5. Activity of ICL in *S. coelicolor* was not found during growth on acetate supplemented medium, possibly due to a permeability problem previously encountered in young mycelia (Cochrane and Peck, 1953), which may explain the lack of glyoxylate bypass activity found by Dekleva and co-workers. The presence of ICDH and ICL reveals the presence of an active glyoxylate bypass in *S. coelicolor*, but it is possibly regulated in a manner different to that in enteric bacteria.

Primary biosynthetic products and intermediates used as precursors for aromatic antibiotics (*e.g.*, novobiocin, chloramphenicol) are produced by the shikimate pathway. Products of this pathway include phenylalanine, tyrosine and tryptophan. A shikimate pathway enzyme, 3-dehydroquinase, has been purified from *S. coelicolor* (White *et al.*, 1990). Interestingly, the enzyme was found to be similar to the quinate catabolising enzymes of *Neurospora crassa* and *Aspergillus nidulans*, rather than the fungal enzyme responsible for formation of 3-dehydroshikimate. Although no growth of *S. coelicolor* was observable on quinate, growth did occur on *p*-hydroxybenzoate, an intermediate in the β-ketoadipate pathway. This indicated the presence of enzymes oxidising aromatic compounds in *S. coelicolor*. The β-ketoadipate pathway is present in aerobic bacteria that utilize aromatic compounds: aromatic rings are opened to give acetyl CoA and succinate via β-ketoadipate.

The study of glycolytic enzymes in *Streptomyces* has been neglected until recently with the purification of phosphoglycerate mutase (PGM) from *S. coelicolor* (White *et al.*, 1992). PGM catalyses the transferrence of the phosphoryl group from the third carbon in 3-phosphoglycerate to give 2-phosphoglycerate. Amino acid sequence comparisons of the *S. coelicolor* PGM revealed high similarity to eukaryotic PGMs; genetic sequences from prokaryotic PGM genes have not been reported. Biosynthetic pathways in *Streptomyces* have also not been studied in great detail. However, the histidine biosynthetic gene cluster of *S. coelicolor* has been cloned and characterized (Capellano *et al.*, 1990; Limauro *et al.*, 1990). Five ORFs within the operon were predicted to encode proteins with sequences similar to the products of the *his* genes of *E. coli*.

Utilization of central metabolic pathways has been shown to alter during the streptomycete growth cycle. For example, the relative utilization of the pathways varies during spore germination and in older *S. griseus* mycelium (Wang *et al.*, 1958b; this section). Because precursors are required for secondary metabolites, central pathway activity must be maintained, but not necessarily at a level comparable to primary metabolism. Ahmed *et al.* (1984) showed that activity of a TCA cycle enzyme, α-ketoglutarate dehydrogenase, decreased in *S. venezuelae* as the mycelia aged, with no activity detectable in older mycelia. Accumulation of α-ketoglutarate occurred in N-limited cultures and was correlated with the loss in enzyme activity: a low α-ketoglutarate producing strain exhibited increasing α-ketoglutarate dehydrogenase activity throughout growth. The excretion of organic acids (also pyruvate) was associated with the complete utilization of nitrogen sources (*e.g.*, $NH_4^+$, but almost complete use of $NO_3^-$), indicating a possible rectifying mechanism for carbon and nitrogen imbalances within cells. Excretion of pyruvate by *S. alboniger* grown on glucose, however, has been linked to a higher activity of glycolytic enzymes not matched by the TCA cycle (Surowitz and Pfister, 1985a). Organic acid production by several other *Streptomyces* has been reported (Cochrane, 1952; 1961; Hockenhull *et al.*, 1954), whereas in the presence of glycine and ammonium the nitrogenous product of α-ketoglutarate metabolism, glutamate, was detected in the cell-free medium of six species (Perlman and O'Brien, 1958).

Studies examining the use of central pathways during secondary metabolism, revealed major use of the EMP pathway during ε-rhodomycinone biosynthesis from radiolabelled glucose in *Streptomyces* sp. C5 (Dekleva and Strohl, 1988a). TCA activity was also high, whereas only a

small contribution was made by the PP pathway. The experiment also showed the metabolic precursor of ε-rhodomycinone to be acetyl-CoA. Contrary to this, chlortetracycline production by *S. aureofaciens* was shown to be correlated with increased utilization of the PP pathway during suppressed glycolysis (Hostalek, 1964). The PP pathway was also mainly used during trehalose production, as shown by $^{13}$C-NMR studies. The advantages of this technique over the radiorespirometry are described by Jeffrey *et al.* (1991).

In addition to the existence of enzymes in pathways similar to the facultative anaerobe, *E. coli*, it has been established that *Streptomyces*, like other aerobic bacteria, possess a cytochrome system. *S. fradiae* and *S. griseus* were shown to contain spectra for cytochromes a, b and c (Niederpruem and Hackett, 1959).

## 1.9 Regulation of primary metabolism

### 1.9.1 Introduction

Primary metabolism in bacteria is regulated to allow organisms to compete efficiently in nature for the limited resources required for growth and reproduction. Control mechanisms include substrate induction, feedback inhibition and repression and catabolite inhibition and repression. In short, substrate induction of an inducible enzyme is dependent on substrate concentration and may control synthesis of one or a group of enzymes. However, constitutive enzymes are produced independently of substrate concentration. Feedback regulation is dependent on the concentration of the end product. Feedback inhibition controls enzyme activity at the level of the enzyme, while repression acts on the genes. Catabolite inhibition and repression mechanisms are similar to those of feedback regulation, but they allow preferential utilization of rapidly metabolizable nutrients.

Examination of regulation of primary metabolism in *Streptomyces* has not paralleled that of the regulation of secondary metabolism. However, because nutrient uptake and utilization affects antibiotic production, a few groups have concentrated on elucidating the responsible regulatory mechanisms, mainly those concerning catabolite repression.

### 1.9.2 Carbon catabolite repression

Catabolite repression of antibiotic biosynthesis is common among *Streptomyces* (section 1.7.1). Few studies have been carried out on the regulation of nutrient metabolism but indications are that regulatory networks exist to favour easily assimilated nutrients that are directed towards growth. Glucose repression of uptake and/or metabolism of several other sugars was observed in *S. coelicolor* (Hodgson, 1982). Subsequently, genes encoding glycerol catabolic enzymes, glycerol kinase and L-glycerol-3-phosphate dehydrogenase, were shown to be induced by glycerol (or possibly glycerol-3-phosphate) and repressed by glucose and other sugars (Seno and Chater, 1983). The genes, *gylA* and *gylB* were found to be organized within an operon, including a non-essential region, *gylX* (Smith and Chater, 1988a). Also included were tandem glycerol-inducible promoters, differing in their sensitivity to glucose repression, and an additional region, *gylR*, that was proposed to encode a *gyl* activator protein (Smith and Chater, 1988b). Similarly, glucose-repressible galactose metabolic genes in *S. lividans* were demonstrated to be present in an operon containing a galactose-inducible promoter and a constitutive promoter (Fornwald *et al.*, 1987). The galactokinase (*galK*) gene from *S. coelicolor* has been cloned (Kendall *et al.*, 1987).

### 1.9.3 Nitrogen catabolite repression

Nitrogen regulation of antibiotic synthesis is also frequently found in *Streptomyces* (section 1.7.1). Consequently, enzymes involved in nitrogen assimilation have been studied. Such enzymes include glutamate dehydrogenase (GDH), glutamine synthase (GS) and glutamate synthase (GOGAT: glutamine-oxoglutarate amino transferase). The comparable enzymes in enterobacteria are responsible for fixing $NH_3$ by reactions 1, 2 and 3 respectively (Reitzer and Magasanik, 1987):

$$\alpha KGA + NH_3 + NADH + H^+ \xrightarrow{GDH} \text{glutamate} + NAD^+ + H_2O \quad 1$$

$$\text{glutamate} + ATP + NH_3 \xrightarrow{GS} \text{glutamine} + ADP + Pi \quad 2$$

$$\text{glutamine} + \alpha KGA \xrightarrow{GOGAT} 2\ \text{glutamate} \quad 3$$

$$\alpha KGA + NH_3 + ATP \longrightarrow \text{glutamate} + ADP + Pi \quad 4$$

Under conditions of low $NH_3$, reaction 1 is inoperable because of the low

affinity of GDH for $NH_3$. Therefore, reaction 3 is preceded by reaction 2 and the net reaction, 4, occurs.

Activities of GDH, GOGAT and GS have been detected in cell extracts of *S. venezuelae* (Shapiro and Vining, 1983), *S. noursei* (Gräfe *et al.*, 1977, cited by Fisher, 1989), and *S. coelicolor* (Fisher, 1989, Fisher and Wray, 1989), and their GS (*glnA*) genes have been shown to be repressible by ammonium. Regulation of GS in *S. cattleya* was shown to be by adenylylation (Paress and Streicher, 1985; Streicher and Tyler, 1981), where an AMP unit is attached to an amino acid residue rendering the enzyme less active (Reitzer and Magasanik, 1987). The gene encoding GS, *glnA*, of *S. coelicolor* has been cloned and sequenced (Wray and Fisher, 1988) and an additional locus, *glnR*, has been found to be required for *glnA* transcription (Wray *et al.*, 1991). GOGAT and GS activities are also present in *S. clavuligerus* and their genes are repressed by ammonium and induced by poor nitrogen sources (Bascaran *et al.*, 1989a; Brana *et al.*, 1986c). Studies on nitrogen-deregulated mutants of *S. clavuligerus* indicated the presence of a regulatory system for control of several nitrogen catabolic enzymes, with GS playing an important role (Bascarán *et al.*, 1989b). Regulatory mechanisms also exist to allow preferential utilization of $NH_4^+$ over $NO_3^-$, as found in *S. venezuelae* (Shapiro and Vining, 1984). However, nitrate reductase is expressed constitutively in this organism.

A nitrogen regulatory system may not affect uptake and utilization of all nitrogen sources. For example, uptake of proline, a nitrogen source, by *S. clavuligerus* occurs in the presence of ammonium (Bascarán *et al.*, 1989a). Later studies showed that proline uptake was mediated by two saturable uptake systems: a proline-specific, high affinity system with low uptake capacity, and a low affinity, higher uptake system subject to amino acid inhibition (Bascaran *et al.*, 1990a). Neither systems were repressed nor inhibited by ammonium. Continuation of proline uptake in the presence of other, more readily utilizable, nitrogen sources may be indicative of a role for proline in osmoregulation. *Staphylococcus aureus* has two proline uptake systems of high and low affinities for proline. The low affinity system shows increased proline transport activity in high osmotic strengths, and may therefore aid in osmoregulation (Townsend and Wilkinson, 1992). A second proline uptake system has been found to function in *S. coelicolor* under conditions of low osmotic potential (Swoboda and Hodgson, 1988).

A relationship between nitrogen regulation of primary metabolism and

secondary metabolism has been demonstrated in *S. avermitilis* which produces avermectins, 16-membered macrolides (section 1.6.2). Avermectins and branched-chain fatty acids share similar precursors and it has been proposed that, since they are synthesized at the same time, competition occurs for these precursors (Novak *et al.*, 1990). Ammonium was shown to decrease yields of both avermectins and lipids (Novak *et al.*, 1992). However, the composition of fatty acids remained unaffected. This is in contrast to the effect of ammonium in *S. fradiae*, where a high concentration inhibited tylosin biosynthesis and decreased the quality of branched-chain fatty acids (Vancura *et al.*, 1987). Valine is a precursor of branched-chain fatty acids and macrolides (Omura *et al.*, 1983), and ammonium was shown to repress and inhibit valine dehydrogenase, the first enzyme of valine catabolism in *S. fradiae*.

## 1.10 Determination of metabolic regulation

Studies on *Streptomyces* have mainly been geared to a further understanding of the switch between primary and secondary metabolism in order to ultimately increase secondary metabolite production. Productivities of commercial strains of both *Streptomyces* and fungi have increased since intial fermentations by use of strain selection and mutagenesis. However, production levels are far short of the theoretical maximum (section 1.3). This leaves much scope for improvement by molecular genetic techniques which should be concentrated on those enzymes most involved in regulation of metabolism.

Bacterial metabolism is the sum product of thousands of chemical reactions, coordinated for efficient and economical utilization of nutrients. A stoichiometric representation of these reactions is given by mass (elemental) balance equations (Cooney *et al.*, 1977; Erickson *et al.*, 1979; Roels, 1980). Mass balance equations relate utilization of substrate, oxygen source and nitrogen source with yield of biomass and product. Substrate, biomass and product are expressed as containing one mole of carbon:

$$CH_{i1}O_{j1} + aNH_3 + bO_2 \longrightarrow y_cCH_{i2}O_{j2}N_{k2} + zCH_{i3}O_{j3}N_{k3} + cH_2O + dCO_2$$

$y_c$ and z are fractions of substrate converted, while a and b are number of moles of $NH_3$ and $O_2$ utilized respectively (Stouthamer and vanVerseveld, 1987). These equations can give an indication of efficiency of substrate

conversion to product by calculation of a Monod yield factor. The yield factor is the weight of biomass produced per unit weight of substrate used (Bushell and Fryday, 1983), and is therefore important in industrial fermentations using high cost substrate materials. Another consideration of major importance is volumetric productivity (Wang *et al.*, 1977), *i.e.*, the flux of substrate to product, and it is this which should be maximised (Kell *et al.*, 1989).

In order to increase flux to a metabolic product, it is necessary to determine the regulation of the flux. Intracellular fluxes are naturally sensed and changed according to metabolic requirements (Crabtree and Newsholme, 1987), but deregulation of the fluxes by genetic manipulation would allow their enhancement (Nimmo and Cohen, 1987). Identification of the regulatory processes obviously requires measurement of such fluxes.

The stoichiometry of metabolic fluxes is represented in mass balance equations. The equations show that input (the substrate) is equivalent to output, especially in terms of moles of carbon. Output is a function of input and metabolic fluxes, but mass balance equations do not give information about these fluxes. However, quantitative measurement of throughputs from input to output serves as a basis by which to determine flux through bacterial central metabolism to the product in question (Holms, 1986).

Throughputs are measures of the amounts of metabolites channeled between growth substrate to product to give a known weight of product (biomass or excreted products). Calculation of throughputs therefore requires knowledge of the metabolic pathways used. A convenient unit to use is $mmol.g^{-1}$ dry weight biomass, *i.e.*, the amount of carbon compound required to produce 1g dry weight of cellular biomass (Holms, 1986). When grown in a glucose-limited medium, 1g of *E. coli* biomass is composed of certain amounts of macromolecules (protein, RNA, DNA, lipids etc.) which, in turn, comprise different monomers (building blocks): amino acids, ribonucleotides, nucleotides, fatty acids and carbohydrates (Morowitz, 1968; 1978; Neidhardt, 1987; Umbarger, 1977). Each of these monomers has as precursor(s) one or more of eight intermediates in the central metabolic pathways (Ingraham *et al.*, 1983). These intermediates/precursors are glucose-6-phosphate, triose phosphate, phosphoglycerate, phospho-*enol*pyruvate, pyruvate, oxaloacetate, oxoglutarate (α-ketoglutarate) and acetyl CoA (figure 1.7).

Construction of a table of monomers and precursors allows computation of the total amount of each intermediate drained from the central pathways for biosynthesis of 1g dry weight biomass (table 1.1). Throughputs ($mmol.g^{-1}$) may then be calculated according to Holms (1986) by sequentially subtracting the drain of intermediates from the amount of input substrate utilized to produce 1g dry weight of biomass. For example, in a continuously fed batch culture *E. coli* ML308 was found to use 8.82mmoles of glucose-6-phosphate to create 1g dry weight biomass with no excreted products except $CO_2$. A throughput diagram was constructed in conjunction with the information on the demands of biosynthesis, *i.e.*, drain of intermediates (Holms, 1986; figure 1.8).

The rate of biomass formation is the specific growth rate, $\mu$, (section 1.5). Since metabolic fluxes are dynamic, and therefore dependent on $\mu$, both throughput and $\mu$ together give a measurement of fluxes ($mmol.g^{-1}.h^{-1}$). In the above example, *E coli* ML308 grew at a growth rate of $0.94h^{-1}$ with the fluxes represented in figure 1.9 (Holms, 1986).

The Holms approach was used to study fluxes in *E. coli* ML308 during growth on acetate. It was shown that operation of the glyoxylate bypass divided the flux of isocitrate such that partitioning of the flux between the enzymes ICDH and ICL was in a ratio of approximately 2:1. ICDH was observed to be involved in control of the flux through the glyoxylate bypass, which agreed with the finding that ICDH activity is tightly regulated (Holms, 1986; 1987). In addition, the calculated ratio of fluxes through ICDH and ICL was very similar to those obtained in earlier studies carried out by Walsh and Koshland (1984). In these earlier studies, the ratios of flux through ICDH and ICL were estimated by comparing the relative proportions of $^{14}CO_2$ evolved by *E. coli* when grown on [1-$^{14}$C]- and [2-$^{14}$C]-acetate, in combination with measurements of endogenous metabolites using $^{13}$C-NMR. This approach resulted in a flux ratio of 2.6. An additional approach by Walsh and Koshland (1984) which involved the kinetic properties of ICDH and ICL, gave a ratio of 2.1.

Use of the Holms method has also identified PEPC as an enzyme involved in the flux to acetate excretion by *E. coli* during growth in glucose-limited cultures (Okungbowa, 1991). Overexpression of the PEPC gene reduced the flux to acetate excretion and increased biomass yield, thus resulting in a more economical conversion of carbon input to biomass at the same growth rate. This was possibly achieved through a decrease in flux from

phosphoenolpyruvate to pyruvate and an increase in flux from phosphoenolpyruvate to oxaloacetate. A higher flux to oxaloacetate would probably result in increased oxidation of acetyl CoA.

The Holms approach to the determination of flux control is global in that the analysis is on a total cellular system. All cellular metabolism is interdependent and fluxes are regulated throughout a cell for optimal efficiency in the available conditions.

An alternative method which is more limited in approach, in that metabolic pathways are considered independently, is Control Analysis based on the Metabolic Control Theory (MCT) of Kacser and Burns (1973) and Heinrich and Rapoport (1974). Other contributors to the theory are mentioned in Kell *et al.* (1989). The MCT is based upon the idea that control of fluxes is not mediated by the activity of only one enzyme in a pathway, as possibly conveyed by the method of Holms (1986), but that all enzymes in a pathway contribute to some extent in controlling the flux through the pathway (Kacser and Burns, 1973). Experimental evidence is now available showing this (Kacser and Porteous, 1987).

The extent to which an enzyme (E) in (or connected to) a pathway controls metabolic flux (J) in steady state conditions is expressed in quantitative terms by the flux-control coefficient:

$$C_E^J = \frac{\delta J/J}{\delta E/E}$$

The flux-control coefficient is defined as the fractional change in flux divided by the fractional change in concentration (or activity) of the enzyme when these changes are made infinitesimally small (Kell *et al.*, 1989). There are as many flux-control coefficients as there are enzymes in the pathway and the greater the magnitiude of the flux-control coefficient (although it cannot exceed unity; see below) the greater is the influence of the enzyme on steady state flux (Kacser and Burns, 1973; Kacser and Porteous, 1987). Determination of flux-control coefficients was shown to be carried out by plotting J versus E on double log paper and obtaining the tangent ($\delta J/\delta E$) to the resulting curve (Kell, 1987; Westerhoff et al., 1984).

Flux-control coefficients are not constants. They will change depending on the conditions (Kell, 1987) and are dependent on the magnitudes of all the flux-control coefficients in the system (pathway; Kacser and Porteous, 1987).

Thus, all flux-control coefficients in a pathway must add up to a finite number. This has been shown by the flux control summation theorem where the sum of all flux-control coefficients in a pathway equals unity:

$$\sum_{i=1}^{n} C_{Ei}^{J} = 1.0$$

(Kacser and Burns, 1973; Kacser and Porteous, 1987). Some enzymes in branched pathways therefore have negative flux-control coefficients since a change in flux through the branch would have a negative effect on the main pathway.

Flux-control coefficients are not just limited to fluxes but if the concentration of an enzyme changes then so too will the concentration of one or more intermediate metabolites (S). Analogous with flux-control coefficients, concentration-control coefficients are defined as the small change in metabolite concentration in response to a small change in enzyme concentration (or activity):

$$C_{E}^{S} = \frac{\delta S/S}{\delta E/E}$$

Again there are as many concentration-control coefficients as there are enzymes in the system and so there is also a concentration-control summation theorem:

$$\sum_{i=1}^{n} C_{Ei}^{S} = 0$$

(Kacser and Porteous, 1987). The outcome of this is that there is no one controlling enzyme but that control of flux and of metabolite concentration is distributed between all the related enzymes. It is possible then to choose rationally which enzyme to concentrate on to increase the required flux (Kell and Westerhoff, 1986).

Control coefficients are global properties of a system. However, single enzymes in a pathway also exhibit local responses to concentrations of substrates, products, effector molecules etc. (X). The fractional change in turnover number (V) of an enzyme divided by the fractional change in metabolite concentration, when all the other parameters and variables are held constant, is the elasticity coefficient:

$$\varepsilon_X^V = \frac{\delta V/V}{\delta X/X}$$

There are as many elasticity coefficients as there are metabolites and effectors interacting with the enzyme (Kacser and Burns, 1973; Kacser and Porteous, 1987).

The response of a change in flux to a change in enzyme concentration is dependent on the elasticity coefficients of the system. The flux-control connectivity theorem, relating flux-control coefficients to elasticities, states that "the sum of the products of the flux-control coefficients of the enzymes in a pathway and their elasticities towards a given metabolite is zero":

$$\sum_{i=1}^{n} C_{Ei}^{J} \cdot \varepsilon_X^V = 0$$

(Kacser and Burns, 1973). Flux-control coefficients may be calculated by matrix methods from the elasticities alone (Fell and Sauro, 1985).

Although the MCT is only applicable to unbranched pathways in steady state conditions, algebraic modifications have been carried out in order to study more complex pathways containing branches or cycles (Sen, 1990; Small and Fell. 1990). Use of the Control Analysis approach is becoming increasingly applied in several different prokaryotic and eukaryotic systems. A review of some is given by Kell *et al.* (1989).

The MCT is complemented by the method of Holms (1986). In one approach the system is a metabolic pathway, in the other the system is cellular. The Holms method allows the definition of a target enzyme for control analysis (Holms 1987) without requiring measurement of all the parameters in the system. However, the metabolic pathways of the organism in question must be known before the approach can be applied. Because of the flux-control summation theorem, the MCT can be used in systems where not all the enzymes in a pathway are known. A black-box approach may be taken where enzymes in a pathway are regarded as a single enzyme and their total contribution to flux control can be estimated. For example, this may be applied to antibiotic biosynthetic pathways providing all the genes are coordinately induced or expressed (Kell *et al.*, 1989). Antibiotics, however, are generally produced in stationary phase, *i.e.*, not during steady state conditions.

Another difference between the two approaches is the consideration of metabolic pools. Holms (1986) considered the contents of metabolic pools to be only a small fraction of the total carbon content of the cell and it was therefore possible to disregard them. However, according to Kacser and Burns (1973), metabolic pools are the links in a system's interactions and were therefore important. Nevertheless, despite the differences in the approaches, global considerations and particular considerations are necessary for analysis of the whole living system (LaPorte, Walsh and Koshland, 1984).

## 1.11 Aims of this work

Physiological studies of *Streptomyces* are necessary for a fuller understanding of the streptomycete life cycle and may also be useful in the attempt to increase production of secondary metabolites (section 1.8.2; 1.10). Recent physiological and genetic studies on *Streptomyces* have concentrated on the switch from primary to secondary metabolism and the control of metabolic fluxes (section 1.8.2). One approach has been to clone appropriate genes by "reverse genetics"; the isolated genes are assumed to have a role in the regulation of fluxes. An alternative approach, which is also complementary to these studies, was undertaken during this work and which involved the quantitation of fluxes during primary and secondary metabolism in *Streptomyces coelicolor*. This was to allow possible identification of enzymes in the central pathways which may have been involved in the regulation of flux to actinorhodin biosynthesis.

*S. coelicolor* produces four secondary metabolites, two of which are coloured: actinorhodin (which is blue under alakaline conditions), undecyl-prodigiosin (red), methylenomycin (if the plasmid SCP1 is present) and calcium-dependent antibiotic (sections 1.6.1; 1.6.5; 1.7.2). Actinorhodin is produced during stationary phase (*i.e.*, not during growth) in batch culture and is therefore a suitable metabolite to consider in a comparision of primary and secondary metabolism. None of the antibiotics produced by *S. coelicolor* have any commercial importance, thus making this strain suitable for academic studies. In addition, *S. coelicolor* is the most genetically well-characterized of the *Streptomyces* and is reported to be typical with respect to streptomycete genetics (section 1.3). Therefore, *S. coelicolor* was proposed to be the best species on which to undertake such physiological studies.

Two methods have been described for the quantitation of metabolic fluxes: the Metabolic Control Theory (MCT) and a method described by Holms (1986) involving the monomeric composition of the organism in question (section 1.10). Both methods require detailed information about central metabolism, little of which is available for *Streptomyces*. However, the detection of central pathway enzyme activities in *Streptomyces* and purification of EMP and TCA cycle enzymes allowed the assumption that, in general, central metabolism of *S. coelicolor* was similar to that of the enteric bacterium, *Escherichia coli*, from which most knowledge of central metabolism has been obtained (section 1.4). Of the two methods, the Holms method for the determination of fluxes requires a more general knowledge of the central and biosynthetic pathways. It was thus possible, and more feasible, to use the Holms method to compute the fluxes of *S. coelicolor* rather than the MCT.

Therefore, the aims of this work were to determine the metabolic fluxes through the central metabolic pathways of *S. coelicolor* during growth and during antibiotic (actinorhodin) production. Estimation of these fluxes required the fulfillment of two additional aims: to grow *S. coelicolor* in a minimal medium suitable for carbon flux analyses and to determine the monomeric composition of the streptomycete biomass. The nature of the work was such that the results are presented in this thesis in three separate chapters. The aims are given in greater detail at the beginning of each chapter.

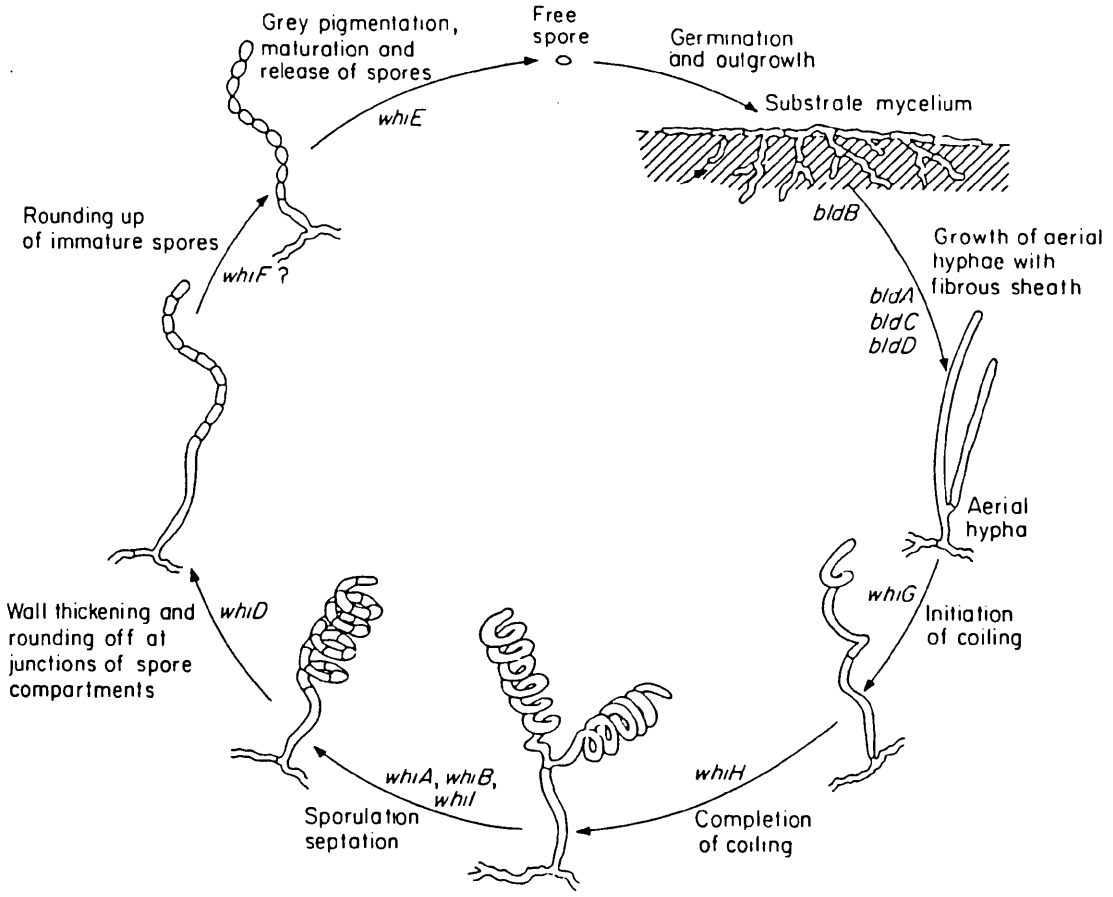



**Figure 1.1. Developmental cycle of *Streptomyces coelicolor*.** (from Chater and Merrick, 1979). The *bld* and *whi* genes involved in each stage of differentiation are described in section 1.7.2.

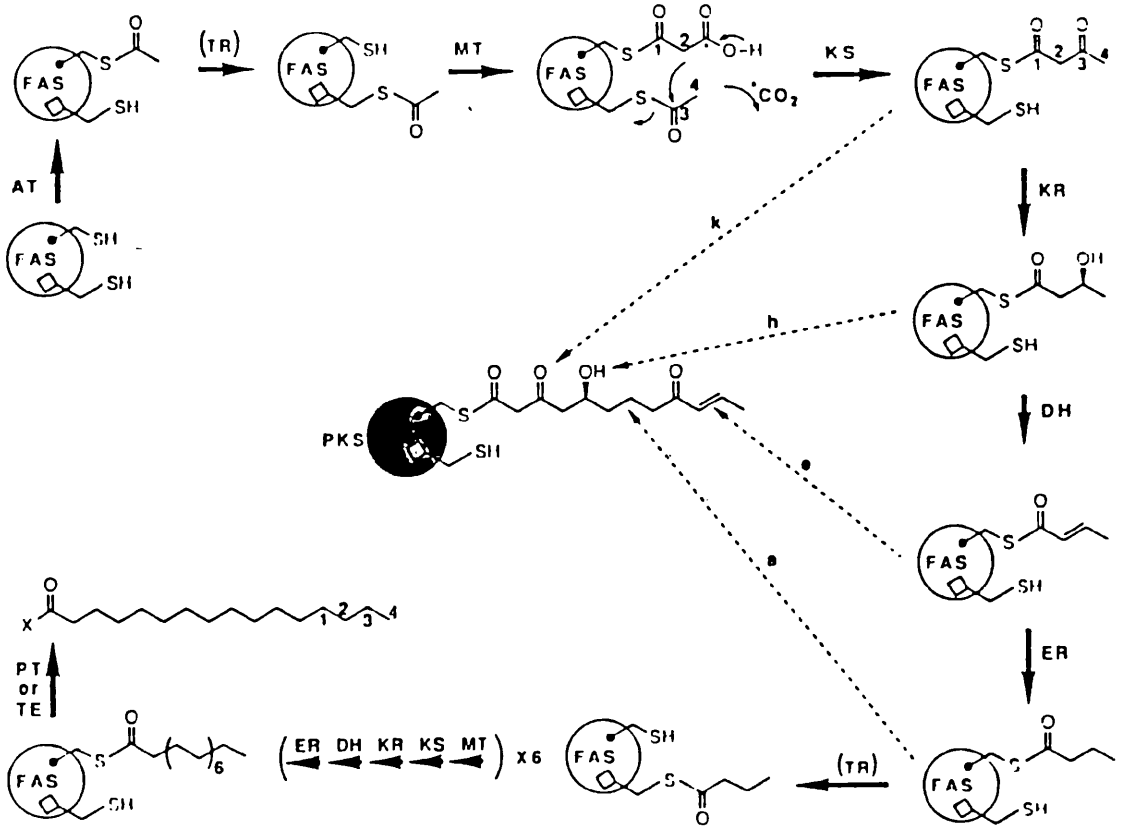



**Figure 1.2. Diagrammatic representation of fatty acid and polyketide biosynthesis** (from Hopwood and Sherman, 1990).
The fatty acid synthase (FAS) and polyketide synthase (PKS), type II, are presented as plain and shaded circles respectively. Both synthases carry two thiol groups, one on the β-ketoacyl synthase (condensing enzyme) (◇) and the other on the acyl carrrier protein (ACP) (●). An example of fatty acid biosynthesis is given by the formation of palmitate, a $C_{16}$ fatty acid.The steps involve the initial transferrence of the acetyl group (the "starter") from acetyl-CoA onto the ACP (AT, acetyl transferase) followed by an acyl transfer reaction (TR) onto the β-ketoacyl synthase. Secondly, a malonyl group (the "extender") is transferred onto the ACP (MT, malonyl transferase) which donates an acetyl residue to the "starter" (KS, β-ketoacyl synthase). The product undergoes reduction (KR, ketoreductase), dehydration (DH, dehydrase) and further reduction (ER, enoyl reductase) and the chain is elongated by repeating the sequence six times. Chain termination occurs by PT (palmityl transferase) or TE (thioesterase) and the final product is palmityl-CoA or free palmitic acid respectively.

Polyketide biosynthesis involves the formation of different functional groups. The letters k, h, e and a denote the possible steps that follow the condensation reaction to give keto, hydroxyl, enoyl or alkyl functions respectively.

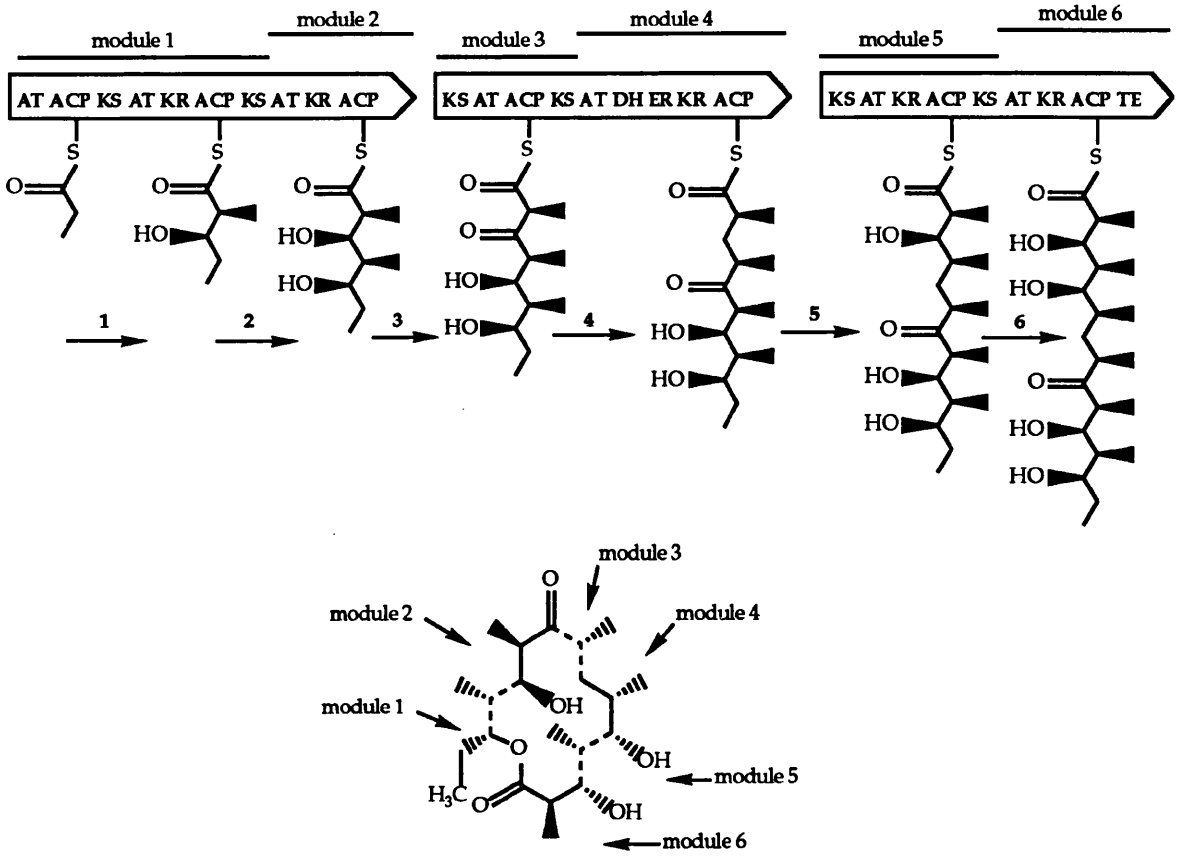
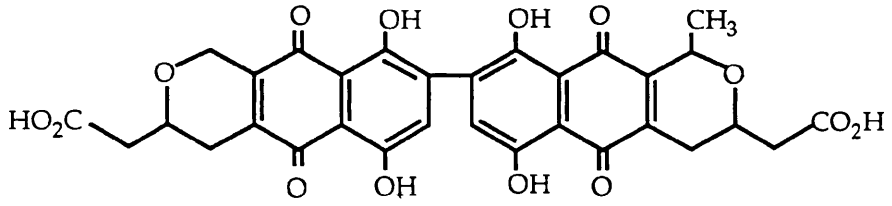
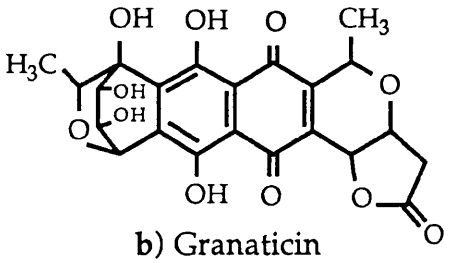


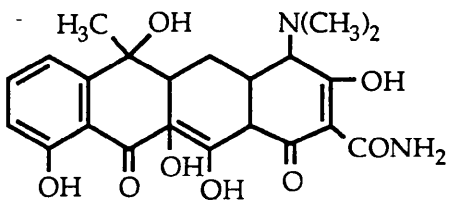

**Figure 1.3. Model for 6-deoxyerythronolide B (the polyketide portion of erythromycin) formation** (from Donadio *et al.*, 1991; Donadio and Katz, 1992).
The erythromycin polyketide synthase of *Saccharopolyspora erythraea* is encoded by a single gene, *eryA* and is a type I PKS. The gene consists of three open reading frames (ORFs) denoted by thick arrows. Each ORF consists of two modules which contain the enzymic activities for each elongation step in the formation of 6-deoxyerythronolide B: AT, acyltransferase; ACP, acyl carrier protein; KS, β-ketoacyl carrier protein synthase; KR, ketoreductase; DH, dehydratase; ER, enoylreductase; TE, thioesterase. Numbered arrows refer to the six elongation steps which occur in a manner similar to that described in figure 1.2 and result in a $C_{15}$ chain. Lactonization between carbons 1 and 13 of the chain results in the formation of 6-deoxyerythronolide B.



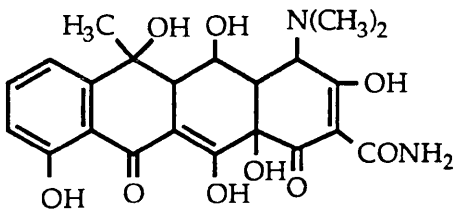
a) Actinorhodin



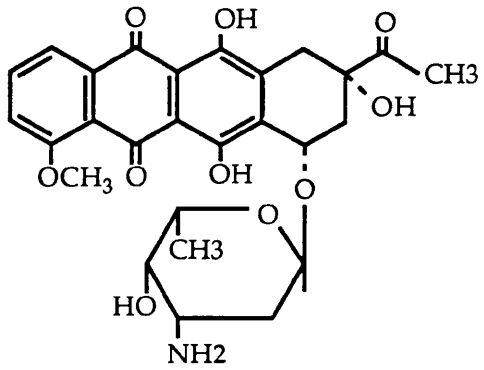
b) Granaticin



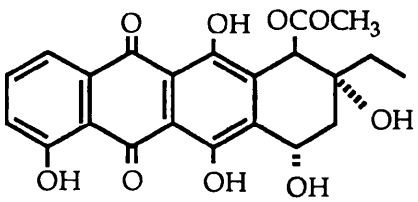
c) Tetracycline



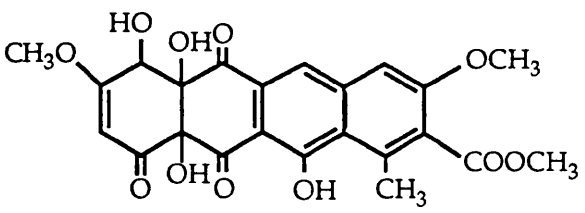
d) Oxytetracycline



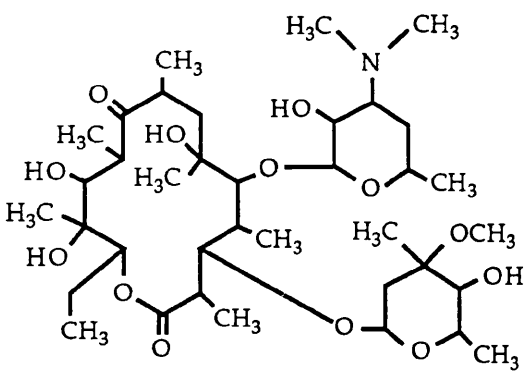
e) Daunomycin



f) ε-Rhodomycinone



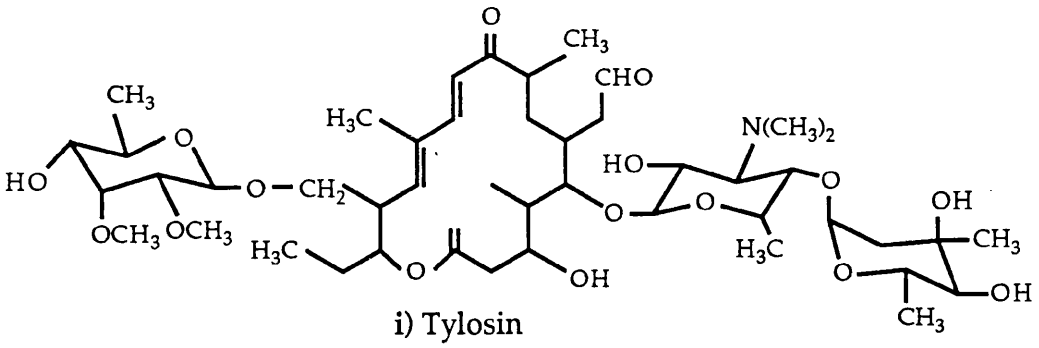
g) Tetracenomycin C



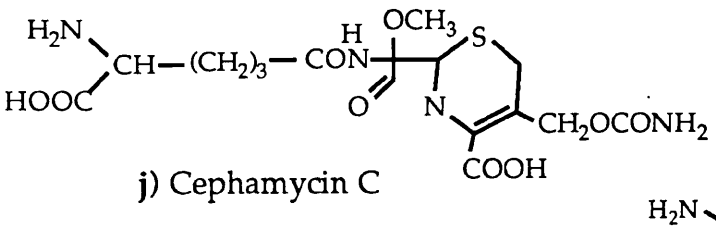
h) Erythromycin

**Figure 1.4. Structures of a selection of antibiotics produced by streptomycete species.**

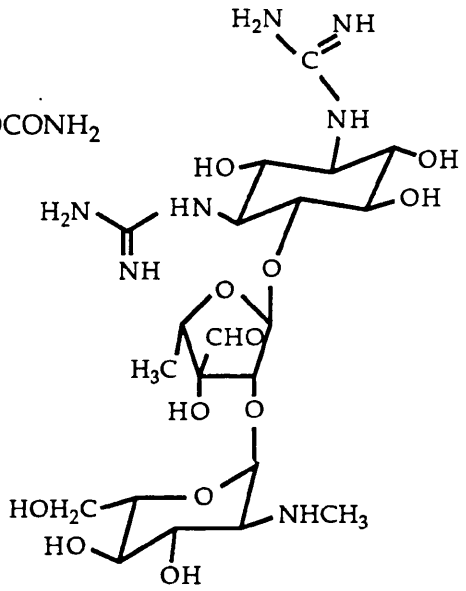
See text for references.



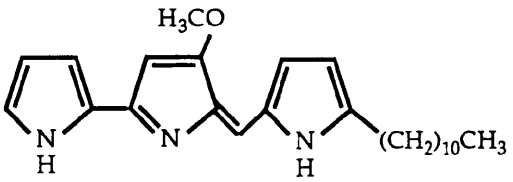
i) Tylosin



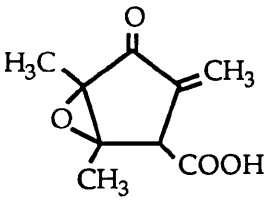
j) Cephamycin C



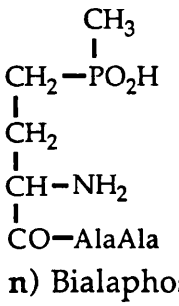
k) Streptomycin



l) Undecylprodigiosin

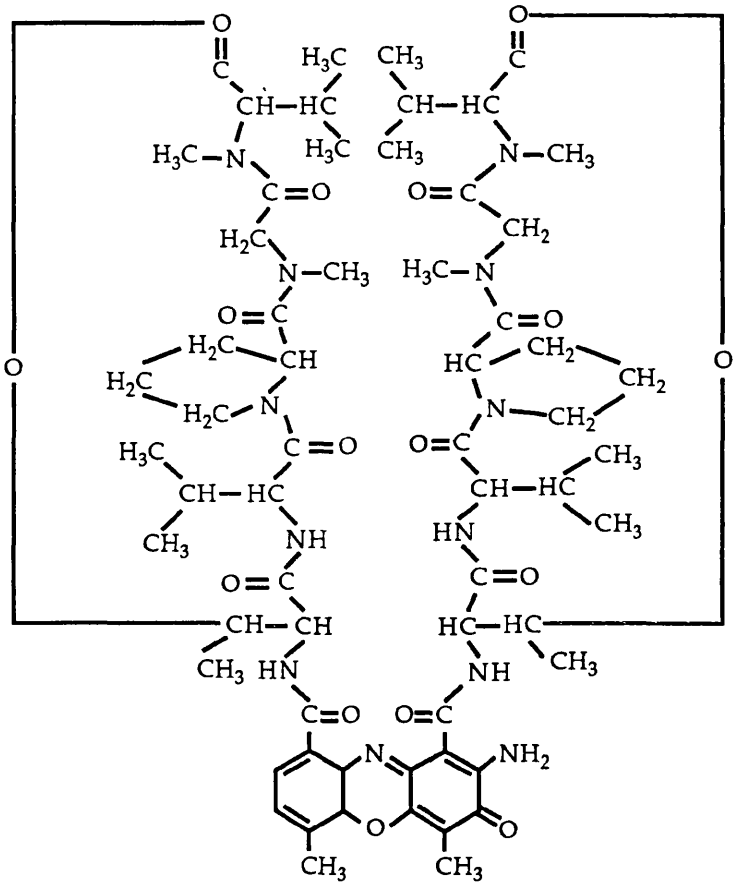


m) Methylenomycin

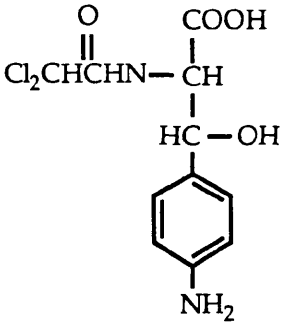


n) Bialaphos

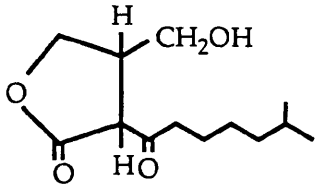
**Figure 1.4** continued.



o) Actinomycin D



p) Chloramphenicol



q) A Factor

**Figure 1.4** continued.

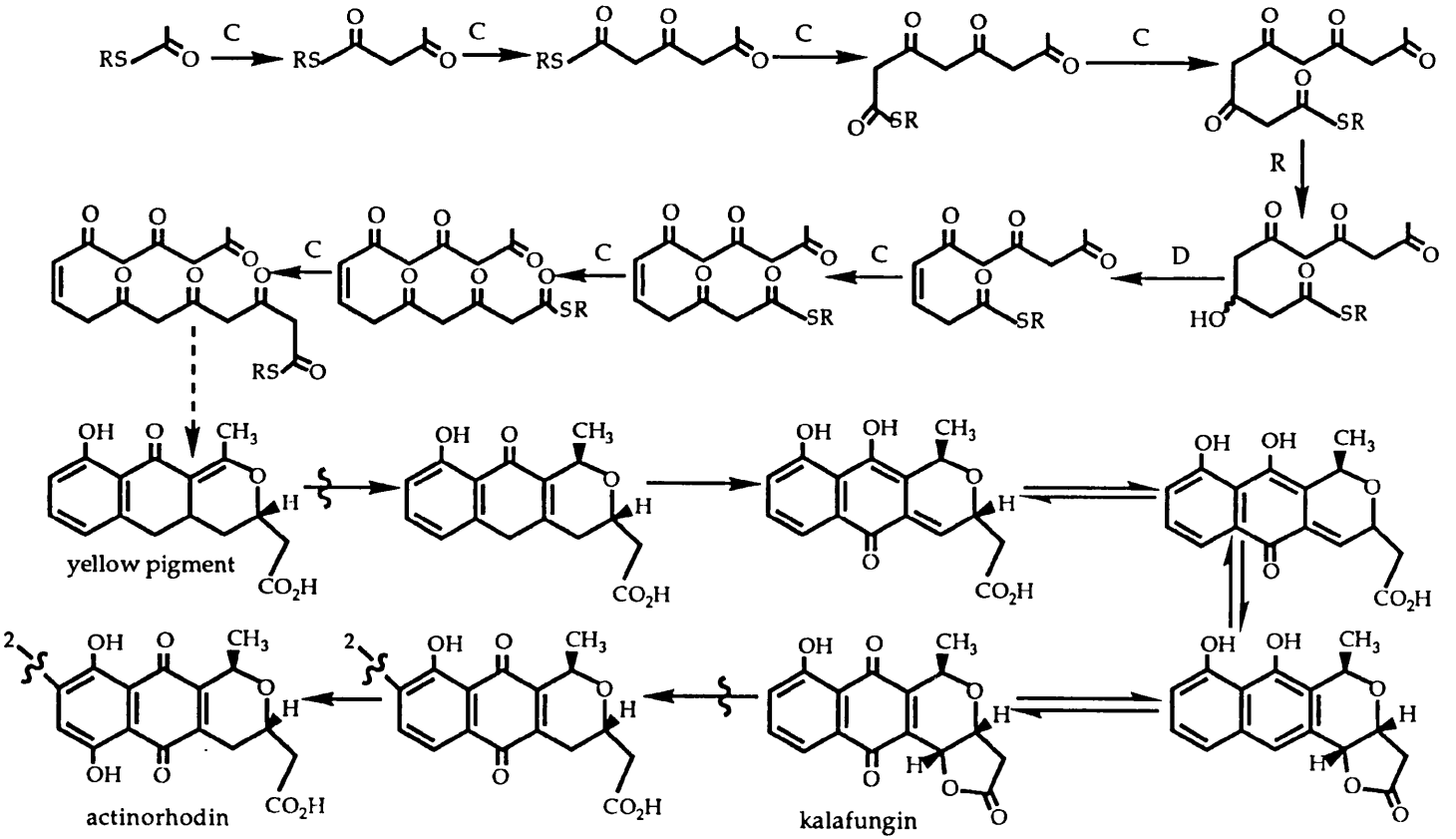



**Figure 1.5. Proposed actinorhodin biosynthetic pathway** (from Sherman et al., 1988; Simpson, 1991).
The pathway was constructed by combining the pathways given in the references cited above. The initial part (up to the dashed arrow) is taken from Sherman *et al.* (1988) and shows the assemblage of the polyketide structure carried out by the polyketide synthase. RS, thiol group of the acyl carrier protein; C, condensation; R, reduction; D, dehydration. The second part of the pathway is a more recent proposal which describes the formation of actinorhodin from a yellow-pigmented precursor via the antibiotic kalafungin (Simpson, 1991).

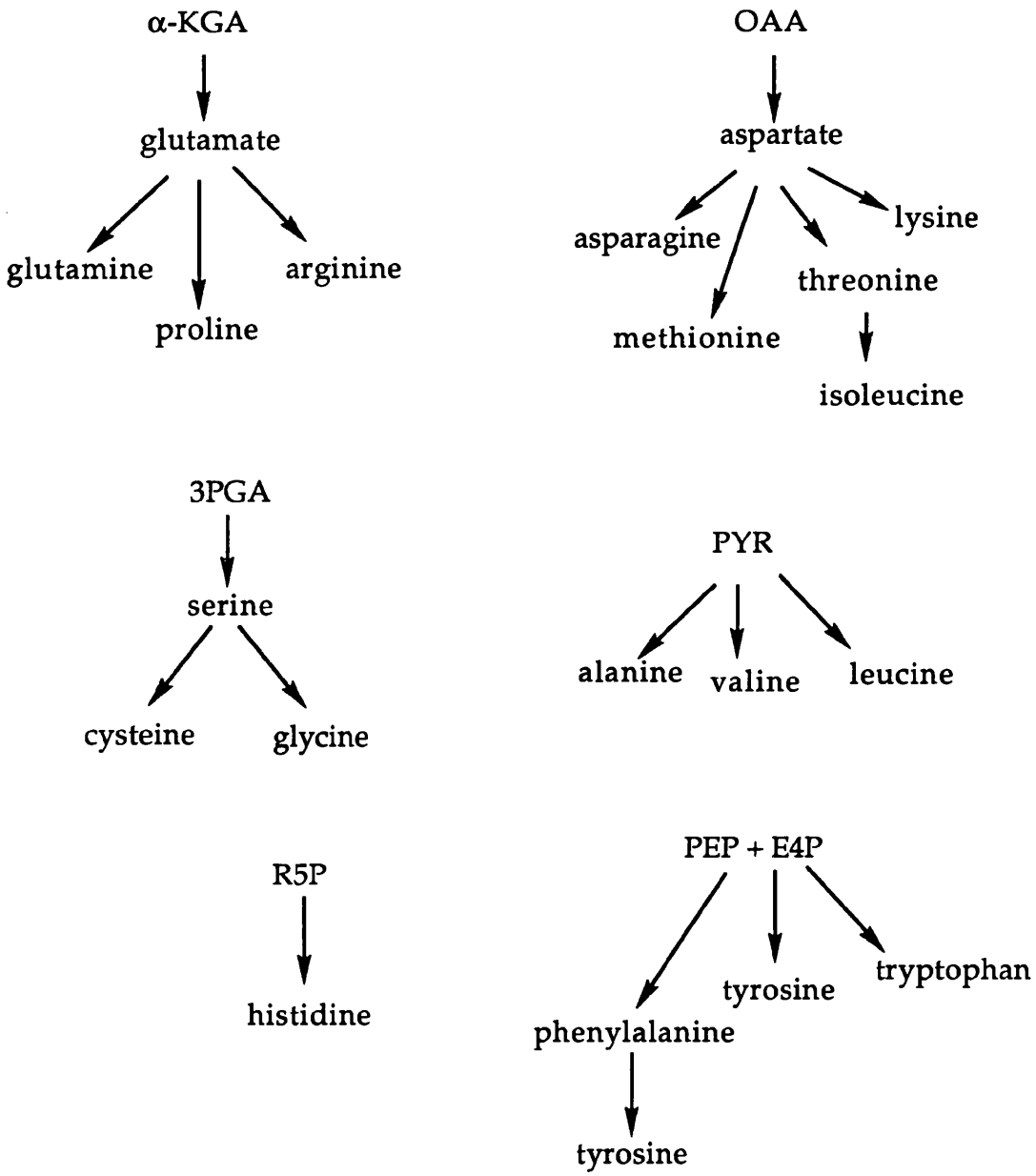



**Figure 1.6. Families of amino acids.**
α–KGA, α-ketoglutarate; OAA oxaloacetate; 3PGA, 3-phosphoglycerate; PYR, pyruvate; R5P, ribulose 5-phosphate; PEP, phospho*enol*pyruvate; E4P, erythrose 4-phosphate.

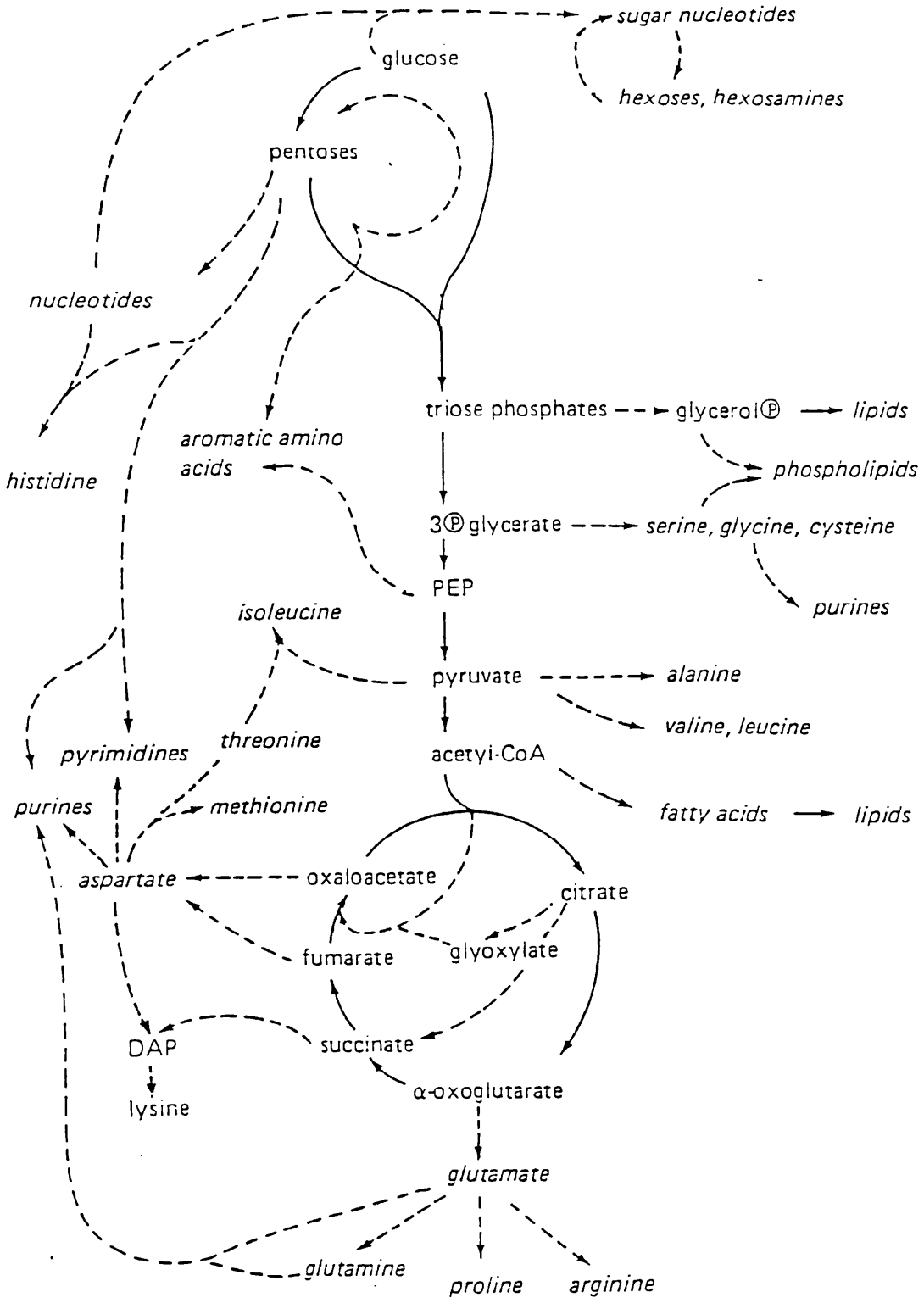



**Figure 1.7. General pattern of monomer synthesis from central metabolites** (from Dawes and Sutherland, 1984).
The central metabolic pathways are indicated by the complete arrows, while the dashed arrows denote the biosynthetic pathways to monomers which stem from central pathway intermediates. PEP, phospho*enol*pyruvate; DAP, diaminopimelic acid. .

**Table 1.1. Amounts of precursors required for biosynthesis of monomers in *E. coli* (from Holms, 1986).**

| monomer content | | central metabolic precursors | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | G6P | TP | PG | PEP | PYR | OAA | OGA | AcCoA |
| alanine | 0.454 | | | | | 0.454 | | | |
| arginine | 0.252 | | | | | | | 0.252 | |
| aspartate | 0.201 | | | | | | 0.201 | | |
| asparagine | 0.101 | | | | | | 0.101 | | |
| cysteine | 0.101 | | | 0.302 | | | | | |
| glutamate | 0.353 | | | | | | | 0.353 | |
| glutamine | 0.201 | | | | | | | 0.201 | |
| glycine | 0.430 | | | 0.430 | | | | | |
| histidine | 0.050 | 0.050 | | | | | | | |
| isoleucine | 0.252 | | | | | 0.252 | 0.252 | | |
| leucine | 0.403 | | | | | 0.806 | | | 0.403 |
| lysine | 0.403 | | | | | 0.403 | 0.403 | | |
| methionine | 0.201 | | | | | -0.201 | 0.201 | | |
| phenylalanine | 0.151 | 0.151 | | | 0.302 | | | | |
| proline | 0.252 | | | | | | | 0.252 | |
| serine | 0.302 | | | 0.302 | | | | | |
| threonine | 0.252 | | | | | | 0.252 | | |
| tryptophan | 0.050 | 0.100 | | 0.050 | 0.050 | | | | |
| tyrosine | 0.101 | 0.101 | | | 0.202 | | | | |
| valine | 0.302 | | | | | 0.604 | | | |
| A | 0.115 | 0.115 | | 0.115 | | | | | |
| dA | 0.024 | 0.024 | | 0.024 | | | | | |
| G | 0.115 | 0.115 | | 0.115 | | | | | |
| dG | 0.024 | 0.024 | | 0.024 | | | | | |
| C | 0.115 | 0.115 | | | | | 0.115 | | |
| dC | 0.024 | 0.024 | | | | | 0.024 | | |
| U | 0.115 | 0.115 | | | | | 0.115 | | |
| dT | 0.024 | 0.024 | | | | | 0.024 | | |
| C16 FA | 0.280 | | | | | | | | 2.240 |
| glycero-phosphate | 0.140 | | 0.140 | | | | | | |
| carbohydrate | 1.026 | 1.026 | | | | | | | |
| total | | 1.984 | 0.140 | 1.362 | 0.554 | 2.318 | 1.688 | 1.058 | 2.643 |

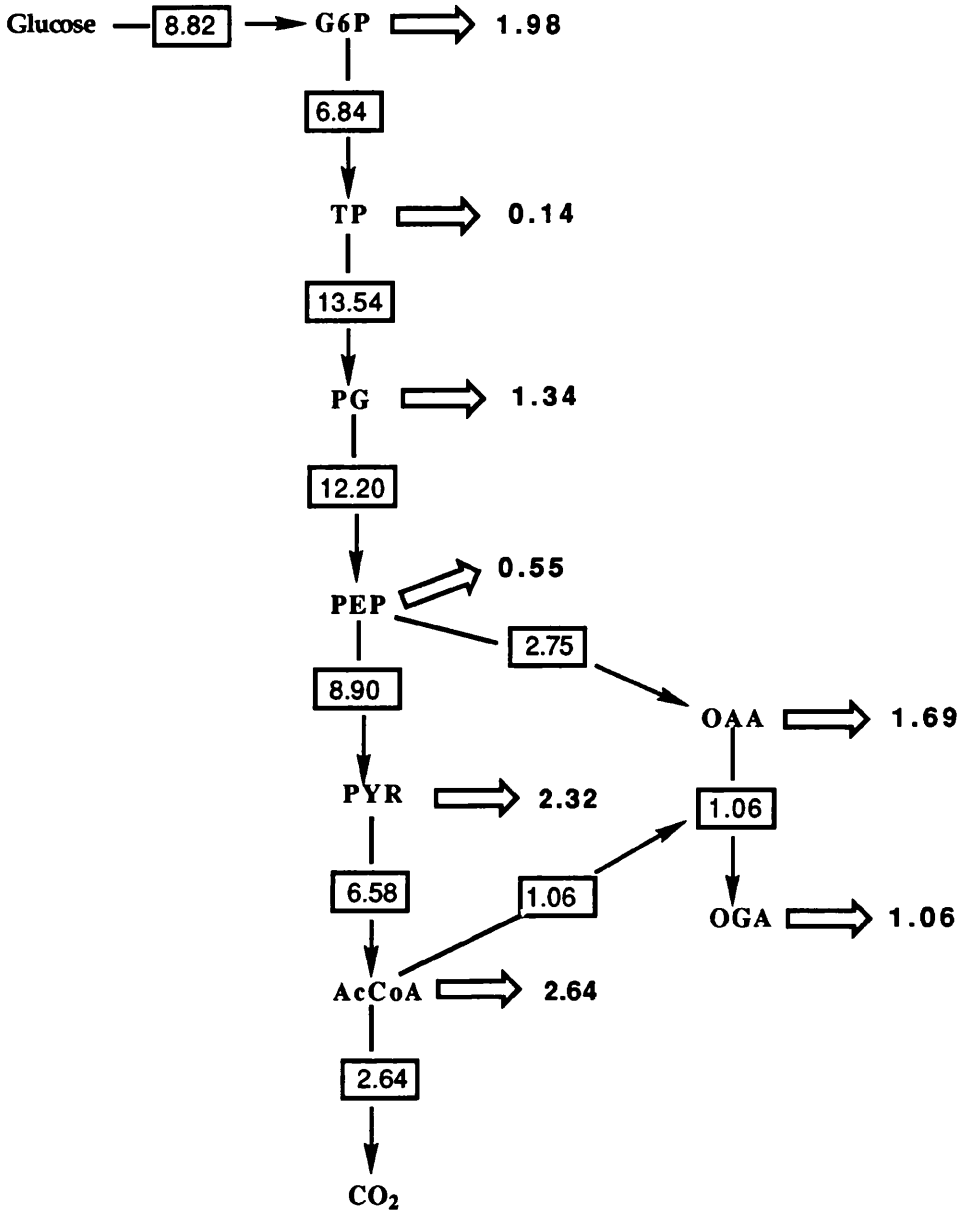



**Figure 1.8. Throughputs through the central metabolic pathways of *E. coli* ML308 grown on glucose 6-phosphate** (from Holms, 1986).
All figures are in mmol.g$^{-1}$. Open arrows show the drain from the central pathway intermediates to biosynthesis.

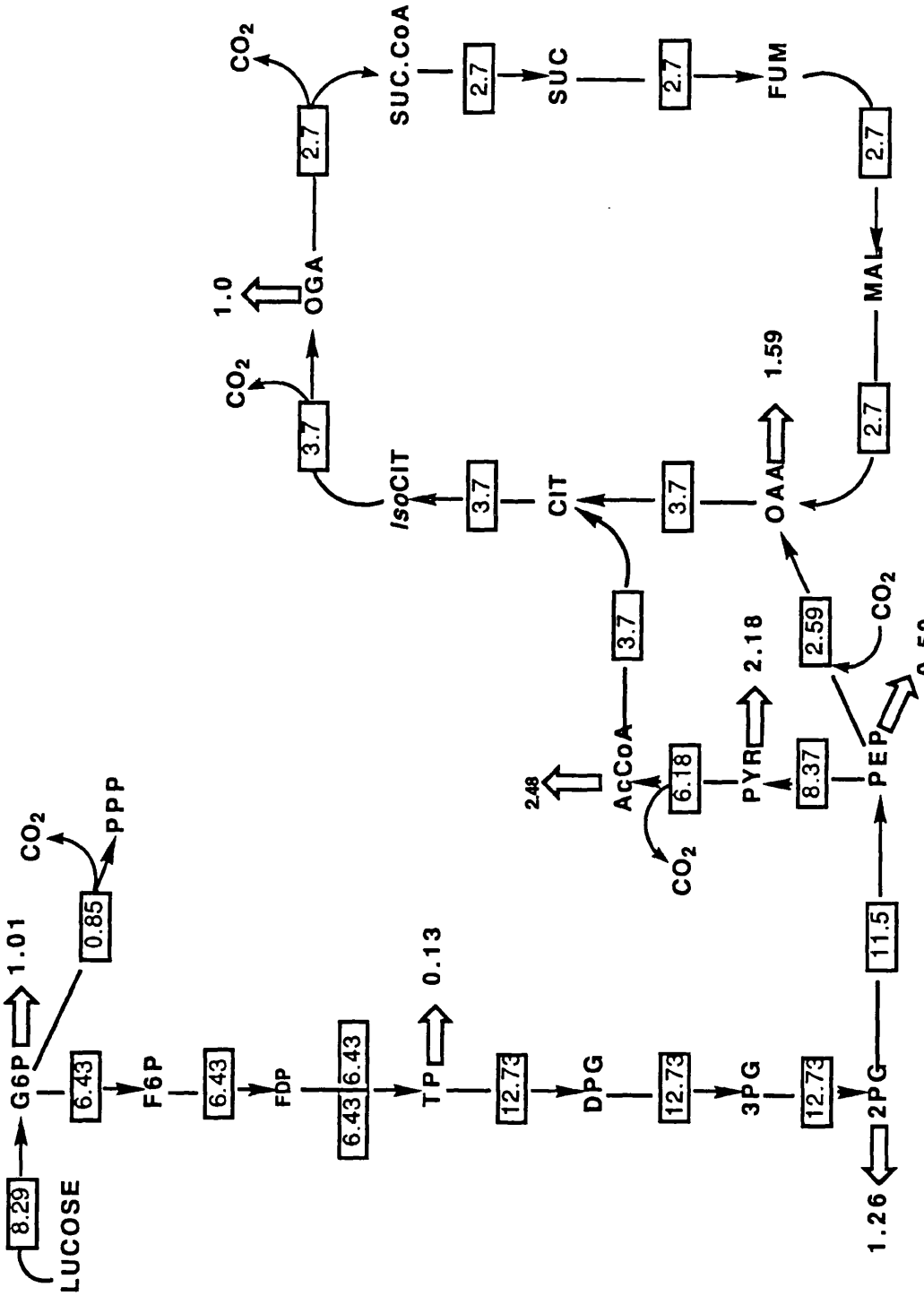



**Figure 1.9.** Fluxes through the central metabolic pathways of *E. coli* (based on that from Holms, 1986). All figures are in mmol.$g^{-1}$.$h^{-1}$.

# Chapter 2

# Materials and Methods

## 2.1 Introduction

This chapter describes the methodology used for the experiments carried out in chapters 3, 4 and 5. Any modifications and necessary additional descriptions of methods are found in the relevant chapters.

Sections 2.4 to 2.8 contain the techniques and materials utilized for maintenance and growth of *Streptomyces coelicolor*. Sections 2.6 and 2.7 also deal with the equipment used for the continuous cultivation studies in the Zentralinstitut für Mikrobiologie und experimentelle Therapie, Jena, FRG.

The assay methods used for determination of the macromolecular and monomeric compositions of *Streptomyces coelicolor* are described in sections 2.9 to 2.13.

## 2.2 Chemicals and Consumables

| | |
|---|---|
| General and AnalaR chemicals | BDH, Koch-Light-Laboratories, Sigma, Fisons, May and Baker. |
| Growth Media | Difco. |
| Junlon | Honeywill and Stein. |
| Antifoam | Sigma. |
| GOD PERID | Boehringer Mannheim GmBH. |
| Gases | British Oxygen Company. |
| Plasticware | Sterilin. |

## 2.3 Equipment

| | |
|---|---|
| Bench centrifuge | Damon/IEC (UH) Ltd., Dunstable, England |
| Temperature-controlled centrifuge | J2-21, Beckman |
| 6 x 250ml rotor | JA-14, Beckman |
| 8 x 50ml rotor | JA-20, Beckman |
| Microfuge | type 5415C, Eppendorf |
| Electronic balance | Oertling |
| Micropipettes | Gilson |

| | |
|---|---|
| Orbital incubator | New Brunswick, New Jersey, USA |
| Sonicator | Dawe Soniprobe, type 7532B |
| Spectrophotometers | Ultrospec 4050, LKB |
| | DU-50 spectrophotometer, Beckman |
| | PU8680 VIS/NIR Kinetic spectrophotometer, Philips |
| Total organic carbon analyzer | model TOC-500, Shimadzu Corporation, Japan |

## 2.4 Microbiological Techniques

### 2.4.1 Strain

Two isogenic strains derived from the wild type strain of *Streptomyces coelicolor* A3(2) were used: *S. coelicolor* 209 and *S. coelicolor* 1147 (Glasgow definition). Strain 209 is a modification of the M145 strain and contains the naturally occuring plasmids SCP1 and SCP2; SCP1 is integrated into the chromosome. Strain 1147 contains the two plasmids SCP1 and SCP2 free in the cytoplasm.

### 2.4.2 Storage of organisms

Because *Streptomyces* grow at a very slow rate, any manipulation is highly sensitive to microbial contamination. Therefore, all manipulations were carried out in a Laminar flow hood. Spores of each strain were obtained from donor soya-mannitol slopes (kindly provided by colleagues at the John Innes Institute, Norwich) which were stored at -20°C.

To acquire spores for pre-germination, new generations of spores were grown from the donor slope spores as follows. The spores were carefully suspended in sterile distilled $H_2O$ with a 25ml syringe and a needle. They were filtered through a cotton wool filter to remove any mycelia and agar. Equal volumes of spore suspension were spread, with a pipette, onto either R2 agar or soya-mannitol agar slopes. The slopes were incubated at 30°C for 9 days and stored at -20°C.

For the procurement of fresh spores, the "master" spores from the donor ATCC slope were suspended in distilled $H_2O$ and filtered as described previously. The suspension was then transferred to a glass universal and spun in the bench centrifuge (3000rpm, 10 min, room temperature (RT)) The supernatant was carefully decanted and the spores resuspended in 10ml of 20% (v/v) glycerol. 1ml aliquots of the "master" spore suspension were stored at -20°C in 1.5ml eppendorf tubes.

## 2.4.3 Preparation of inocula

### 2.4.3.1 Pre-germinated spores.

Spores were pre-germinated following the method of Hodgson (1982), as follows:

TX Buffer:

| | |
|---|---|
| TRIS-HCl, pH8.3 | 0.05M |
| Triton X-100 | $0.01g.l^{-1}$ |

Sterilized by autoclaving.

Hirsch Complex Medium (HCM), per litre:

| | |
|---|---|
| $MgSO_4.7H_2O$ | 0.10g |
| $CaCl_2$ | 0.02g. |
| Tris-HCl, pH7.3 | 0.1M |
| Triton X-100 | 0.01g. |
| Yeast Extract | 10.0g |
| Casamino Acids | 10.0g |

Sterilized by autoclaving.

Spores were gently scraped from one slope by the addition of 10ml of $dH_2O$ with a 10ml pipette (or a 25ml glass syringe and needle), transferred to a universal bottle and centrifuged in the bench centrifuge (3000rpm, 10min, RT). The pelleted spores were resuspended in 0.3ml of TX buffer, followed by incubation at 50°C for 10 minutes ("heat shock"); cooling was carried out under a cold tap. 10ml of HCM was then added and the spore suspension was shaken for two hours at 30°C in a rotary shaker. The spores were re-

centrifuged (3000rpm, 10min, RT), resuspended in 20% (v/v) glycerol and stored at -20°C. These spores were used to inoculate shake flasks only.

### 2.4.3.2 Fresh spores.

Fresh spores were obtained by inoculating one soya-agar plate with a loopful of spores from the frozen "master" suspension and incubating the plate at 30°C for 8 to 10 days. The spores from this "master" plate were then suspended in 10ml $H_2O$, and filtered, as before, and equal volumes (300µl to 500µl) spread onto 10 soya plates ("working" plates). The plates were incubated at 30°C for 8 to 10 days. Spores from the "working" plates were suspended in sterile $H_2O$ and used to inoculate the fermenter.

For the inoculation of flasks, the fresh spores were resuspended in 20% (v/v) glycerol as for pre-germinated spores. 350µl of a spore suspension from one plate gave approximately $1x10^7$ spores.$ml^{-1}$ when inoculated into 50ml of medium in a 250ml Erlenmeyer flask.

### 2.4.4 Spore Counts

Dilutions of spores in $H_2O$ in the range $1x10^{-1}$ to $1x10^{-8}$ were added to molten Emersons agar and mixed. The plates were incubated at 30°C for 3 days and the colonies counted. The concentration of spores was calculated by multiplying the number of colonies by the appropriate dilution factor.

A Spore Counting Chamber (0.01mm, 1/400$mm^2$, Thorna Fein-Optik, Bad Blankenburg, FRG) was used in Jena. The number of spores in 16 squares were counted and divided by 16 to obtain a mean value (MV). The concentration of spores was calculated as follows:

$$MV \times 4x10^7 \times \text{dilution factor (DF)} = \text{number of spores per ml.}$$

The numbers of viable spores were checked using serial dilutions (as described above).

## 2.5 Media

The following media were prepared using distilled $H_2O$ and autoclaved for 15 minutes at 15p.s.i., unless stated otherwise. Media for the continuous culture experiments were autoclaved in 10litre or 20litre vessels for 1 hour.

### 2.5.1 New Minimal Media minus Junlon (NMM-J)

This glucose, salts minimal media was based on the medium used by Hobbs *et al.* (1989) but did not contain the polyacrylate junlon. Junlon was found to be difficult to remove from the cells and also interfered with carbon balances and some colorimetric assays (see section 4.3.3). The medium without junlon allowed the production of both undecylprodigiosin and actinorhodin and was, therefore, useful in the study of the physiology of *S. coelicolor*.

Per litre:

| | | |
|---|---|---|
| $NaNO_3$ | 4.5g | (52.9mM) |
| NaCl | 5.0g | (85.6mM) |
| $Na_2SO_4$ | 5.0g. | (35.2mM) |
| $MgSO_4.7H_2O$ | 1.0g. | (4.1mM) |
| $CaCl_2.6H_2O$ | 0.83g | (3.8mM) |
| $ZnSO_4$ | 0.01g. | (0.06mM) |
| Tris | 1.2g | (9.9mM) |
| Trace Salts | 1ml. | |

The medium was adjusted to pH7.0 with HCl and autoclaved.

Trace Salts solution (per litre):

| | | |
|---|---|---|
| $ZnCl_2$ | 2.00g | (14.7mM) |
| $MnCl_2.4H_2O$ | 1.00g | (5.1mM) |
| $H_3BO_3$ | 0.30g | (4.8mM) |
| $CuCl_2.2H_2O$ | 0.43g | (2.5mM) |
| $Na_2MoO_4.2H_2O$ | 0.25g | (1.0mM) |
| $FeCl_3$ | 8.70g. | (53.6mM) |
| NaI | 0.42g | (2.8mM) |

The following components were made up as separate solutions and autoclaved individually in order to prevent caramelization and precipitation, respectively, at high temperatures. Addition to the medium took place at the time of inoculation.

| | | |
|---|---|---|
| Glucose | $4.0g.L^{-1}$ | (22.2mM) |
| $K_2HPO_4$, pH7.0 | $1.5g.L^{-1}$ | (8.6mM) |

## 2.5.2 *Streptomyces* R2 Agar

This agar was used for the regeneration of spores for pre-germination. It was prepared in two parts:

R2/A (per litre):

| | | |
|---|---|---|
| $K_2SO_4$ | 0.5g | (2.9mM) |
| $MgCl_2.6H_2O$ | 20.2g | (99.4mM) |
| $CaCl_2.2H_2O$ | 5.9g | (40.1mM) |
| Glucose | 20.0g | (111.1mM) |
| Proline | 6.0g | (52.1mM) |
| Casamino Acids | 0.2g | |
| Trace elements | 4ml | |
| Agar | 44.0g | |

R2/B (per litre):

| | | |
|---|---|---|
| MOPS, pH7.4 | 11.5g | (54.9mM) |
| Yeast Extract | 10.0g | |
| Sucrose | 203.0g | (593.1mM) |

Both parts were autoclaved separately.

100ml of R2/B were heated to approximately 55°C and added to 100ml of molten R2/A at the same temperature. 1ml of 1% (v/v) $KH_2PO_4$ was added per 200ml final volume.

### 2.5.3 Soya Agar

Soya agar was used for the regeneration of fresh spores and for the determination and examination of colony forming units from continuous culture samples, in Jena.

Per litre:

| | |
|---|---|
| Soya flour | 20g |
| Mannitol | 20g |
| agar | 16g |

The components were dissolved in 1litre of tap water and autoclaved.

### 2.5.4 Emersons Agar

This agar was inoculated with appropriately diluted samples from cultures for the determination of colony forming units.

Emersons agar (Difco) 41.5g

The agar was dissolved in 1litre distilled $H_2O$ and autoclaved.

## 2.6 Growth Apparatus

### 2.6.1 Shake Flasks

250ml Erlenmeyer flasks with cotton wool bungs containing 50ml of medium were inoculated with spores to give a spore concentration of approximately $1x10^7$ spores.ml$^{-1}$ medium (*i.e.*, $5x10^8$ spores per flask). The flasks were shaken in an orbital incubator with a 5cm base plate throw, at 30°C and 250rpm. To obtain dispersion in NMM-J medium, stainless steel springs or glass beads (2-3mm diameter, 1g per flask) were added to the flasks. Baffled flasks (Chemistry Department, University of Glasgow) were also used, with and without stainless steel springs. Initially, "Repelcote" (2,4-dichloromethylsilane; Hopkin and Williams) was used in some flasks to give a silicon coating in an attempt to prevent mycelia growing on the sides of the vessels.

## 2.6.2 Fermenters

For the majority of batch cultivations, an 8litre Bioengineering fermenter (Bioengineering, CH-8636 Wald, Switzerland, type L1523) was used. The configurations are shown in figure 2.1. Air was supplied via a separate compressor through a rotameter ($1vol.vol^{-1}.min^{-1}$, $10litres.min^{-1}$ maximum). Temperature was regulated by steam and mains cold water at pressures of 40psi and 4bar respectively, and was measured by a platinum resistance thermometer, maintaining temperature (30°C) constant to +/- 0.3°C. Sterilization of the fermenter and medium occurred *in situ* (122°C, 20 minutes). Sterility was maintained throughout inoculation by a ring of flaming ethanol around the porthole, and throughout sampling, via the steam-sterilizable delivery valve; this was steamed for 10 minutes before and for 5 minutes after sampling.

Continuous cultivation of *Streptomyces coelicolor* took place in the facilities available in the Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, FRG.. A 600ml chemostat with working volume of 460ml (maintained by the use of a weir) was set up in a water bath, maintained at 30°C, as illustrated in figure 2.2. All glass connections were coated with vacuum grease and the vessel was internally coated with methylsilane to reduce wall growth. Sterilization of the chemostat and tubing was by autoclave; connections were sterilised by a disinfectant containing 80ml of 96% ethanol and 16ml of conc. HCl. Inoculation of the chemostat was from a round bottomed flask which could be attached to an outlet port via rubber and glass tubing. Medium flowed from 10litre reservoirs through tubing of diameter 3mm. Medium flow was kept constant by use of a peristaltic pump (PGH Mechanik, JENA, FRG), with changeable cog wheels for alteration of flow rate. The glass stirrer (with 15x8mm blade) was turned by a motor (VEM VEB Elmo, Hartha, FRG 1279.2/1) at 2800rpm and aeration was maintained at $0.1litre.ml^{-1}.h^{-1}$ (Roth and Noack, 1982). Sampling was via an outlet port for small volumes (up to 6ml) and via the effluent tube (into ice) for larger volumes.

## 2.7 Measurement of Growth

### 2.7.1 Wet Weight

Samples taken from the fermenter or pooled cultures from 2 to 3 flasks were harvested in 250ml centrifuge buckets (12400g, 20min, 4°C). The pellets were washed (12400g,10 min, 4°C) with ice-cold $dH_2O$ and transferred with a small volume of chilled $dH_2O$ to a pre-weighed 30ml Beckman centrifuge tube. The pellets were centrifuged (12100g, 10 min, 4°C) and the tubes inverted and left to drain. The pellet was weighed and the weight expressed as $g.l^{-1}$.

### 2.7.2 Dry Weight

Three methods were used in the attempt to obtain reproducible dry weights. All samples were weighed in triplicate and expressed as $g.l^{-1}$ culture.

#### 2.7.2.1 Filtration

10ml samples of cultures were pipetted onto pre-dried, pre-weighed filters, on a scintered glass funnel connected to a conical flask with side-arm, and washed three times with distilled $H_2O$. Filters were dried in a microwave oven (10 minutes at defrost and left to cool for 20 minutes with silica gel present to ensure no rehydration of the filters).

#### 2.7.2.2 Foil cups

10ml samples were harvested in 15ml corex tubes (121000g (with inserts), 10min, 4°C), and the pellets were resuspended in 10ml $dH_2O$ and re-centrifuged. The pellets were then transferred with 10ml $dH_2O$ to pre-dried, pre-weighed foil cups (aluminium foil was moulded around the bottom of a universal bottle to give a cup) and dried at 80°C to constant weight.

#### 2.7.2.3 Corex tubes

20ml samples were harvested in pre-dried, pre-weighed 30ml corex tubes (12100g (with inserts), 10min, 4°C). The pellets were resuspended in 20ml

ice-cold $H_2O$ and re-centrifuged. The remaining pellets were resuspended in 20ml ice-cold $H_2O$ and dried at 80°C to constant weight.

### 2.7.3 Carbon Content

Culture samples (1ml to 1.5ml) were spun (14000rpm (maximum speed in microfuge), 5 min), washed twice in 1ml of buffer (10mM $KH_2PO_4$, pH7.0) and resuspended in a final volume of 200µl. Total organic carbon was measured using a Shimadzu Carbon Analyser.

Samples were carried on high purity air at 150ml.min$^{-1}$ either through the total carbon (TC) or inorganic carbon (IC) reaction tubes. The TC combustion tube was filled with a platinum oxidation catalyst and was maintained at 680°C. Samples (10µl) passing through the tube were oxidised or combusted into $CO_2$, which was detected by a non-dispersive infra-red gas analyzer (NDIR). The NDIR emitted a peak shaped detection signal and the peak area was directly proportional to the TC concentration in the sample. The area was compared to that of a previously injected standard solution (potassium hydrogen phthalate, 400ppm of carbon).

The IC reaction tube was filled with a strong acid liquid with a halogen absorbing silver salt, at 150°C, and only inorganic carbon compounds were combusted to $CO_2$. The $CO_2$ was detected as for TC, but using the IC standard which was a mixture of sodium hydrogen carbonate and sodium carbonate (400ppm of carbon).

Five replicate samples were injected into the TOCA and the mean was calculated. Total organic carbon (TOC) concentration was calculated by:

$$TOC = TC - IC.$$

Dry weight measurements were calculated by multiplying TOC figures by the appropriate dilution factor to give g.l$^{-1}$.

### 2.7.4 Glucose Estimation

Growth was also measured by determining the utilization of glucose in the medium. Glucose concentrations were estimated by two methods:

2.7.4.1 GOD PERID

The test kit (Boehringer Mannheim) contained glucose oxidase (GOD) and peroxidase (PERID). In the assay, the two enzymes act sequentially to form a green coloured complex, measurable at 610nm.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS*} \text{ (pale yellow)} \xrightarrow{\text{PERID}} \text{coloured complex} + H_2O$$

* di-ammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonate).

The reagent solution was prepared according to the manufacturers directions and stored at 4°C. 900µl of the reagent were added to 100µl of appropriately diluted samples (1 in 40 dilution of samples up to 50 hours of growth, 1 in 20 dilution thereafter), vortexed and incubated at 25°C for 25 minutes. The absorbance was measured at 610nm. Standards were included in the range of 0µg to 100µg using the glucose standard solution in the kit.

2.7.4.2 Glucose Oxidase Probe

Glucose estimations in Jena were carried out using a glucose probe with glucose oxidase (E.C. 0.1.1.3.4.) fixed between two membranes (Glukometer 01, Akademie der Wissenschaften der DDR, Zentrum für Wissenschaftlichen Ceradeban). The principle reaction is the same as in the GOD PERID test kit, resulting in the production of gluconic acid and $H_2O_2$.

The enzyme operated in the following buffer (per litre):

| | |
|---|---|
| $KH_2PO_4$ | 1.80g |
| $Na_2HPO_4.2H_2O$ | 9.60g. |
| NaCl | 2.30g |
| $K_2C_2O_4.H_2O$ | 0.30g |
| $NaN_3$ | 0.06g |
| NaF | 0.30g |

pH 7.15 to 7.30.

The signal from the probe was adjusted to zero with 2ml of buffer. Glucose concentrations were then determined by adding 50µl of sample to the buffer. A standard solution of $8g.l^{-1}$ glucose in buffer was used. The standard gave a reading of 80.0 allowing glucose samples to be read at 10 times the final concentration.

## 2.8 Gas Exchange Measurement

The $CO_2$ in the effluent gas from the Bioengineering fermenter was measured in one experiment.

The air flow into the fermenter was regulated by means of a Brooks Mass Flow Controller (models 5850 and 5878, 100% = $15litre.min^{-1}$). The $CO_2$ concentration in the effluent air was measured, as a percentage, by a $CO_2$ Analyzer (Analytical Development Co. Ltd., Hoddesdon, England, type 877). The analyzer has a split-beam detection system for $CO_2$, utilizing the absorption of infra-red by the C=O bonds which are stretching and vibrating.

The analyzer depends on a constant stream of air passing through, at $1litre.min^{-1}$, from which the $CO_2$ has been removed by soda-lime for base-line comparison. Calibration of the analyzer was carried out using 0.8% $CO_2$ in oxygen-free nitrogen and the output was adjusted to zero with oxygen-free nitrogen. The analyzer was set at 0% - 1.0% and calibrated every 24 hours.

## 2.9 Fractionation of Biomass

Two methods were used to extract the macromolecular components of *S. coelicolor*.

**a)** A modification of the method originally described by Schneider *et al.* (1950) incorporating the initial procedure of Ogur and Rosen (1950).
**b)** A modification of the method described by Schmidt and Thannhauser (1945).

Both of these methods were reviewed by Hutchison and Munro (1961).

### 2.9.1 Method 1

A pellet of known wet weight was resuspended in ice-cold 0.2N PCA to a concentration of 100mg wet weight.ml$^{-1}$, and left overnight at 4°C. Centrifugation (12100g, 20min, 4°C) separated the cold PCA fraction (the supernatant) from the pellet. The supernatant was decanted into a plastic 25ml Sterilin universal bottle and the pellet was resuspended in the same volume of 0.5N PCA and digested at 70°C for 30 min. The suspension was centrifuged and the hot PCA fraction decanted into a Sterilin universal bottle. Addition of an equal volume of 0.5N NaOH and incubation (37°C, overnight), followed by centrifugation, resulted in the alkali fraction. The pellet was resuspended in the same volume of distilled $H_2O$ giving the residue fraction, which, depending on particle size, was subjected to sonication (Dawe Soniprobe, type 7532B, output 6, two 20sec pulses).

### 2.9.2 Method 2

A biomass pellet of known wet weight was resuspended in ice cold, distilled $H_2O$ to a concentration of 50mg.ml$^{-1}$. 5ml of resuspended biomass (*i.e.* 250mg) were transferred to a 15ml corex tube. 1.25ml ice-cold 30% (v/v) PCA (3N) were added and the tube was left on ice for 10 min. The sample was centrifuged (9800g, 15min, 4°C) and the supernatant (supernatant 1) decanted into a Sterilin universal tube. The pellet was resuspended in 5ml 0.3N KOH and incubated at 37°C for 90 min, after which it was chilled on ice for 10 min. 1.25ml chilled 2N PCA were then added to the solution which was left for a further 10 min on ice. Re-centrifugation resulted in supernatant 2, and the residual pellet was digested in 5ml 0.5N PCA at 70°C for 60 min, chilled on ice for 10 min and re-centrifuged to give supernatant 3. Resuspension of the pellet in 5ml 0.5N NaOH with incubation at 37°C overnight and re-centrifugation resulted in supernatant 4. Supernatant 5 was the outcome of addition of 5ml distilled $H_2O$ to the pellet, followed by sonication.

These methods are summarised in the Addendum.

## 2.10 Estimation of Macromolecular Composition

The macromolecular composition of the biomass pellet was determined by means of spectrophotometric assays on the above fractions.

## 2.10.1 Carbohydrate Estimation

This was carried out by the method of Shields and Burnett (1960) using anthrone which detects polyglucose.

Anthrone

The reagent was prepared by adding 500ml conc. $H_2SO_4$ to 200ml of chilled $H_2O$ in a vessel on ice. 1g of anthrone was then dissolved in the solution which was stored in a brown bottle. The reagent was stable for one week at room temperature.

5ml of anthrone reagent was pipetted into large boiling tubes standing on ice. When cold, 100µl of sample or standard were added slowly and the solution vortexed carefully. The tubes were placed in a boiling water bath for exactly 10 minutes and then cooled in an ice-water slurry. The absorbance was measured at 620nm. Standards of 0µg to 100µg of glucose (using a standard solution of $1mg.ml^{-1}$ glucose in 0.2N PCA) gave a linear curve.

## 2.10.2 DNA Estimation

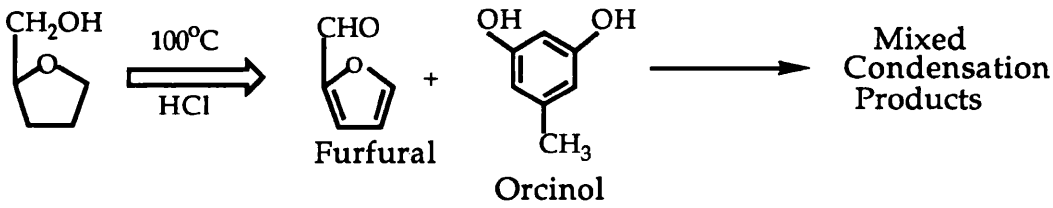
The method used was a modification of that described by Burton (1956). The diphenylamine in the reagent is thought to combine with deoxypentoses formed from the hydrolysis of purine residues. Inorganic phosphate is liberated in the early stages of the reaction, presumably from the phosphate-phosphate bridges between adjacent purine residues.

The reagent was made of 500ml glacial acetic acid, 7.5ml conc. $H_2SO_4$ and 7.5g diphenylamine, and stored in a brown bottle. The addition of 100µl of 1.6% acetaldehyde (stored at 4°C) to 20ml of Burton reagent resulted in the working reagent, which was prepared immediately before use.

2ml of the working Burton Reagent were added to 1ml of sample and vortexed. The tubes were covered with foil and left to stand at 30°C for 18 to 48 hours. The absorbance was read at 600nm. Standards of 0µg to 100µg of DNA per ml assay ($1mg.ml^{-1}$ DNA in 0.5N PCA) were also included.

## 2.10.3 RNA Estimation

Brown (1946) described a method using orcinol to detect the pentoses formed from the hydrolysis of RNA. Strong acid converted the pentoses to furfural which produced a green colour on reacting with orcinol in a condensation reaction with ferric chloride ($FeCl_3$) as the catalyst. Interference by hexoses was eliminated because the supernatants containing RNA were devoid of carbohydrates.




The two reagents were: 0.03% (w/v) $FeCl_3$ in conc. HCl, and 20% (w/v) orcinol (3,5-Dihydroxytoluene) in 95% (v/v) ethanol (prepared daily).

3ml of 0.03% $FeCl_3$ in conc. HCl were added to 3ml of sample (diluted with 0.5N PCA), followed by an addition of 200µl of 20% Orcinol. The solutions were mixed thoroughly and placed in a vigorously boiling water bath for 30 minutes (with glass tear-drops to prevent evaporation and loss of volume). The tubes were cooled in an ice-water slurry and the absorbance was measured at 665nm. Standards were obtained from a stock solution of $1mg.ml^{-1}$ RNA in 0.5N PCA and gave a linear curve in the range of 0µg to 150µg of RNA.

## 2.10.4 Protein Estimation

Three methods were used to determine protein content of the biomass and one to determine the total amount of amino groups.

### 2.10.4.1 Bradford Assay (Bradford, 1976)

The assay worked on the principle of protein dye-binding.

The Bradford reagent was prepared by dissolving 100mg Coomassie Brilliant Blue G-250 in 50ml lab-grade ethanol (95%; v/v), adding 100ml 85% (v/v) orthophosphoric acid and then deionised $dH_2O$ to give a final volume of

1000ml. Filtration was required to remove excess dye. The addition of deionised $dH_2O$ was important to obtain the copper coloured reagent, otherwise the newly prepared reagent would remain blue after filtration.

2.5ml of Bradford reagent were added to 50µl of sample and vortexed. The absorbance was measured at 595nm after 5 min and before 20 min. Standards of 0µg to 100µg per assay were used from a stock of 1mg. $ml^{-1}$ BSA in 0.5N NaOH.

2.10.4.2 Lowry Method (Lowry *et al.*, 1951)

This method depended on the formation of $Cu^{1+}$ ions from the reaction of $Cu^{2+}$ ions in alkaline conditions, followed by the reduction of the phosphomolybdic phosphotungstic reagent by the Cu treated protein. The presence of the reagent enhanced the colour formation by the Cu treated protein (the biuret reaction).

For the assay, the reagents were made up in three parts:

Solution A
2% (w/v) $Na_2CO_3$ dissolved in 0.1N NaOH

Solution B
0.5% (w/v) $CuSO_4.5H_2O$ dissolved in 1% (w/v) sodium tartrate

Solution C
Folin-Ciocalteau reagent, diluted 1 part in 4 with $dH_2O$

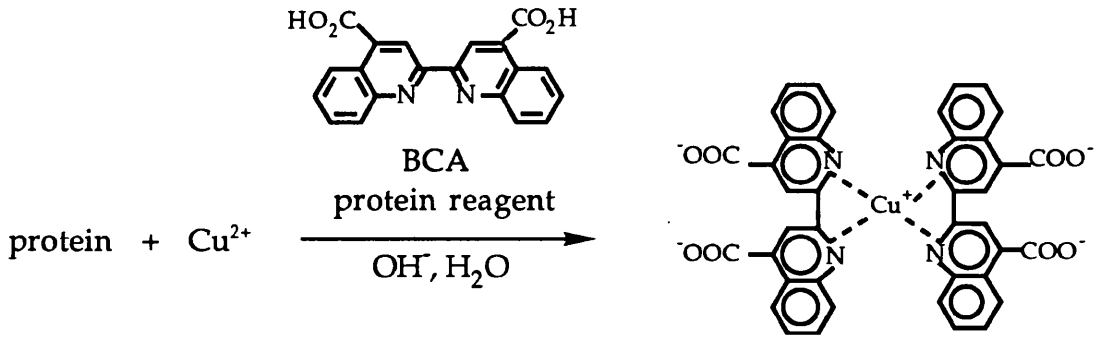
50ml of solution A were mixed with 1ml of solution B immediately before use.

5ml of the mixed solution were added to 500µl of sample, or standard, and left for 10 minutes at room temperature (RT). 5ml of solution C were then added and immediately mixed well. The tubes were left at RT for 30 minutes and the absorbance measured at 750nm. Standards of 25µg to 500µg of BSA were used to obtain a standard curve.

A microassay was used more frequently where the above volumes were divided by 5.

2.10.4.3 Bicinchoninic Acid (Smith *et al.*, 1985)

The principle of the assay was similar to that of the Lowry method in that the formation of $Cu^{1+}$ ions from the biuret reaction is utilized. However, the reagent used in this assay was one containing Bicinchoninic Acid (BCA) which is highly specific for $Cu^{1+}$ ions. The purple product formed exhibits a strong absorbance at 562nm.




The reagent was made up in two parts using deionised $dH_2O$:

Solution A

| | |
|---|---|
| BCA-$Na_2$ | 1% (w/v) |
| $Na_2CO_3$ | 2% (w/v) |
| $Na_2$ tartrate | 0.16% (w/v) |
| NaOH | 0.4% (w/v) |
| $NaHCO_3$ | 0.95%(w/v) |

The pH of solution A was adjusted to pH11.25 by the addition of NaOH (50%).

Solution B

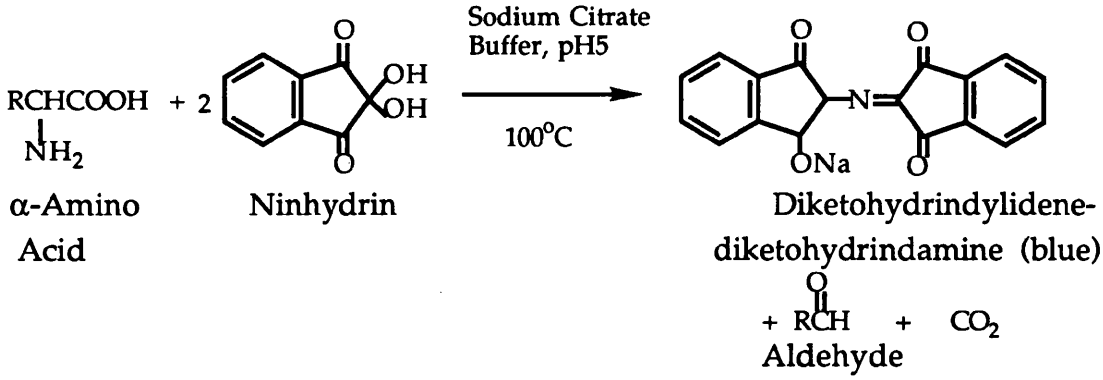
| | |
|---|---|
| $CuSO_4.5H_2O$ | 4% (w/v) |

Both solutions were stable indefinitely, whereas the standard working reagent, composed of the solutions A and B in the ratio 100:2 (v/v) respectively, was stable for one week at RT.

The assay was carried out by adding 20 volumes of the standard working reagent to 1 volume of sample and incubating at RT for 2 hours. The absorbance was then read at 562nm. Standards of 0µg to 100µg of BSA were used to construct a standard curve.

2.10.4.4 Ninhydrin Assay (Moore and Stein, 1948)

The assay is based on the reaction between amino acids and ninhydrin which results in a blue coloured product (known as diketohydrindylidine-diketohydrindamine), $CO_2$ and an aldehyde.




The blue product has an absorbance maximum at 570nm.

A citrate buffer was prepared by dissolving 4.3g of citric acid and 8.7g of sodium citrate in 250ml of $dH_2O$ and adjusting the pH to 5.0. The buffer was stored at 4°C. Prior to use, 400mg of stannous chloride ($SnCl_2.2H_2O$) was dissolved in the citrate buffer with gentle heating. Also prior to use, a solution of 2g of Ninhydrin in 50ml of methoxymethanol was prepared. In addition, 50%(v/v) propan-1-ol was required.

For the determination of total amino groups, 900µl of 13.5N NaOH were added to 100µl of sample in Nalgene tubes and autoclaved for 20 minutes. Once cooled to RT, 2ml of glacial acetic acid were added and the tubes vortexed to neutralise the solution of digested protein.

The ninhydrin and stannous chloride solutions were mixed together in a ratio of 1:1 (v/v), and 1.5ml of the resulting reagent were added to 0.5ml of digested sample. The tubes were placed in a boiling water bath for 5 minutes and then cooled. Subsequently, 8ml of 50% (v/v) propan-1-ol were added and vortexed. After being left at RT for 30 minutes, the absorbance was measured at 570nm. A blank containing $dH_2O$ and standards (usually 500µl of 1mM leucine, because the curve was linear) were included in the assay.

2.10.5 Phosphate Estimation (Nakamura, 1950; cited by Chiba and Sugahara, 1957, based on Allen, 1940)

Phosphate determinations were carried out to verify the nucleic acid values estimated by the Burton and Orcinol methods, and for the estimation of teichoic acid. Utilisation of phosphate from the growth media was also determined by this assay.

The reagents required for this assay were 8.3% (w/v) ammonium molybdate, 1% (w/v) amidol (2,4-diaminophenol hydrochloride) dissolved in 20% (w/v) sodium metabisulphite and 30% (v/v) hydrogen peroxide.

For the estimation of total phosphate groups, 100µl of conc. $H_2SO_4$ were added to 100µl of sample or standard followed by heating at 180°C in a dry block for 60 minutes. Once cooled to room temperature (RT), 0.5ml of $H_2O_2$ was added and the digestion was continued for 30 minutes. If coloured following digestion, an additional 0.5ml of $H_2O_2$ was required with another 30 minute digestion step. However if clear, 4.2ml of $dH_2O$ and 0.2ml of ammonium molybdate were added when the tubes were cool, and vortexed. 0.4ml of amidol reagent were then added, vortexed, and left to stand at RT for 60 minutes. The absorbance was measured at 620nm. A stock solution of $1.36g.l^{-1}$ $KH_2PO_4$ (10mM) was used for standards.

For inorganic phosphate determination, 100µl of conc. $H_2SO_4$ were added to 100µl of sample or standard and the assay method was followed from the addition of 4.2ml of $dH_2O$, thereby eliminating the digestion steps.

## 2.11 Estimation of Actinorhodin

An equal volume of 1N NaOH was added to a sample of culture, to increase the pH to greater than pH12; the sample was then spun in a microfuge for 5min. The absorbance of the blue supernatant was initially read at 523nm and the concentration of actinorhodin was calculated using an extinction coefficient (ε) of 15135 (Horinouchi and Beppu, 1984). In later experiments, the absorbance of the supernatant was read at 605nm. An absorbance of 1.0 was equivalent to a concentration of $120\mu g.ml^{-1}$ (DA Hopwood, personal communication; Magnolo *et al.*, 1992). Actinorhodin has a molecular weight of 624.

## 2.12 Estimation of Monomeric Composition

The monomeric composition was determined and compared with the data obtained from the macromolecular composition.

### 2.12.1 Nucleotide Calculation

The nucleotide composition of the biomass was calculated from the DNA and RNA estimations based on the finding that *Streptomyces* DNA is, on average, 70% GC rich (Pridham and Tresner, 1974) and the rRNA and tRNA sequences, which make up 96% of total RNA, are approximately 60% GC rich (see appendix B).

### 2.12.2 Amino Acid Determination

#### 2.12.2.1 Hydrolysis

Hydrolysis was carried out following the method of Hirs *et al.* (1954) using biomass at a concentration of 100mg of wet weight.$ml^{-1}$ (approximately 200nmoles amino acid. 500$\mu l^{-1}$).

500µl of each sample were transferred (in triplicate) into acid-washed, thick-walled test-tubes by a positive displacement pipette (SMI Digital Adjust Micro/Pettor, American Dade, USA). 100µl of 2mM taurine or 2mM ε-n-amino-caproic acid were added, as internal standards. Following freezing at -80°C using a mixture of dry ice and methanol, the tubes were drawn in a flame of a mixture of natural gas and oxygen. 600µl of conc. HCl were then added to the samples to give a final concentration of 6N HCl. Nitrogen was blown through the samples to remove the oxygen and the samples were re-frozen. The tubes were sealed under vacuum (using an Edwards High Vacuum Pump, type E550) and one tube from each triplicate was left at 106°C for either 24h, 48h or 72h. This ensured the total hydrolysis of each amino acid since amino acids such as valine and isoleucine are released slowly under these conditions.

### 2.12.2.2 Equipment

Hydrolyzed amino acids were analysed on a Philips PU4100 Liquid Chromatograph with a JASCO 820-FP Intelligent Spectrofluorimeter and a Philips PU 6030 DCU. Connected to the Chromatograph was a Philips P3202 Microcomputer using PU6000 software and a PR0 9CM073 VDU. The column used was a reversed-phase column (Brownlee Spheri-5 RP-18, 5µm, 220mm x 4.6mm). Guard columns were of the type RP-18, 15mm x 3.2mm (Brownlee).

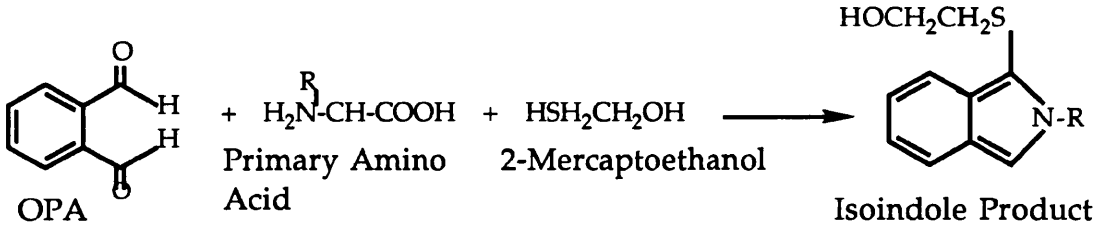
### 2.12.2.3 Principle

The reversed-phase column contained a silica stationary phase which was bonded to octadecylsilyl groups ($C_{18}$) to produce a hydrophobic surface. Solute retention was due to hydrophobic interactions with solutes eluted in order of increasing hydrophobicity (Lim, 1987). Increasing the polar aqueous phase through the column increased the retention of the solutes.

According to Jarrett *et al.* (1986), separation of amino acids by reversed-phase high pressure chromatography required the use of two solvents, an aqueous solvent and a non-polar solvent. This combination, with the column, allowed the acidic and polar amino acids to be eluted first, followed by those with short alkyl side chains, and then the hydrophobic amino acids. Increasing the ionic strength increased the retention times of the acidic amino acids to a greater extent than the neutral amino acids; the basic amino acids were only slightly, if at all, affected. Decreasing the pH also led to longer retention times of the acidic amino acids because of the strengthening interactions with the $C_{18}$ stationary phase; fluorescence was decreased with a decrease in pH (Cooper *et al.*, 1984).

### 2.12.2.4 Derivatization

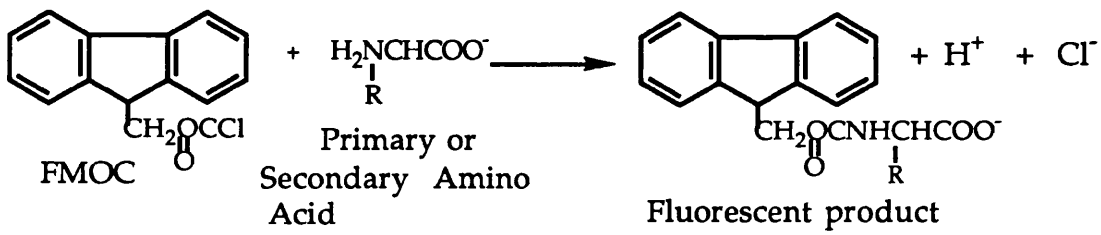
Primary amino acids were determined by pre-column derivatization with OPA (*o*-phthalaldehyde). OPA reacted with the primary amino acids, in the presence of 2-mercaptoethanol (or mercaptopropionic acid), to give highly fluorescent, thio-substituted isoindole derivatives (Simons and Johnson, 1976) which were detected at excitation and emmission wavelengths of 230nm and 455nm respectively:




The OPA reagent was prepared in a borate buffer as follows:

50mg of OPA in 1ml of methanol
40µl of mercaptoethanol (or mercaptopropionate)
10ml of potassium borate buffer (0.2M, pH9.5).
4µl of mercaptoethanol (or mercaptopropionate) were added weekly to maintain activity.

Secondary amino acids were derivatized (pre-column) using FMOC-Cl (9-fluorenylmethyl chloroformate) which reacts with both primary and secondary amino acids (Moye and Boning, 1979). The fluorescent fluorenylmethyl products, which were detected at excitation and emmission wavelengths of 260nm and 313nm respectively (Einarsson *et al.*, 1983), were formed as follows:




The reagent used was composed of 155mg of FMOC-Cl in 40ml of acetone.

Derivatization of the hydrolyzed samples were carried out in one or two steps:

| | |
|---|---|
| Single step derivatization: | 20µl of sample or standard |
| (primary amino acids) | 20µl of OPA (or FMOC-Cl). |
| | Wait 1 minute, |
| | Inject 20µl. |

| | |
|---|---|
| Double step derivatization: | 20µl of sample |
| (primary **and/or** secondary | 20µl of OPA. |
| amino acids) | Wait 1minute. |
| | 20µl of FMOC-Cl. |
| | Wait 1 minute. |
| | Inject 20µl. |

(The determination of secondary amino acids alone required the presence of the Borate buffer, and the OPA was used to react with the primary amino acids. Excitation and emission wavelenths of 260nm and 313nm respectively permitted the sole detection of proline).

### 2.12.2.5 Separation

Three methods were used for the separation and quantitation of the amino acids present in the hydrolyzed samples. Each procedure contained a solvent gradient method for the separation of the majority of the amino acid peaks, and a single (isocratic) solvent method for the separation of two peaks not resolved by the gradient method.

A mixture of amino acid standards of 0.1mM each was run prior to any samples during the gradient methods and used to determine the amino acid concentrations. The standard mixture contained aspartate, glutamate, serine, histidine, glycine, threonine, alanine, taurine, arginine, tyrosine, valine, methionine, isoleucine, phenylalanine, leucine and lysine. The concentration of the standards run for the isocratic methods were 0.5mM.

Amino acid concentrations in the samples were calculated using the following equation :

$$\frac{\text{peak area sample}}{\text{peak area standard}} \times 0.1\text{mM} \times \text{dilution factor}$$

Method 1a

This was a gradient method for the detection of both primary and secondary amino acids, based on that described by Schuster (1988).

Solvent A:
0.02N $CH_3COONa$ (sodium acetate)
0.5% (v/v) THF (tetrahydrofuran)
pH7.0

Solvent B:
90%AcN (acetonitrile)
10% (v/v) 20mM $CH_3COONa$
pH7.0.

Gradient method:

| Segment | Minutes | Solvent A | Solvent B |
|---|---|---|---|
| 1 | 0-3 | 100% | 0% |
| 2 | 3-36 | 55% | 45% |
| 3 | 36-37 | 5% | 95% |
| 4 | 37-42 | 5% | 95% |
| 5 | 42-43 | 100% | 0% |
| 6 | 43-58 | 100% | 0% |

Flow rate: 1500µl.min$^{-1}$

| Detection: | initial | (at 27 min.) | (at 40 min.) |
|---|---|---|---|
| Excitation: | 230nm | 260nm | 230nm |
| Emmission: | 455nm | 313nm | 455nm |

Derivatization: double step (OPA-3-MPA and FMOC-Cl)
Run time: 35 minutes.

Method 1b
This was an isocratic method used in parallel with method 1a. The run time of 20 minutes allowed resolution of the first six amino acids (asp to thr, appropriate standards were used at 0.5mM).

Solvent:
90% (v/v) of 20mM $CH_3COONa$, pH6.0
10% (v/v) AcN
1.0% (v/v) THF.

| | |
|---|---|
| Flow rate: | 1000µl.min$^{-1}$ |
| Detection: | 340nm |
| Derivatization: | single step, OPA/Borate with mercaptoethanol. |
| Run time: | 20 minutes. |

Method 2a

This gradient method was based on method 1a but resolved the peaks from taurine onwards.

solvent A:
0.02N $CH_3COONa$
0.5% THF
pH7.0

solvent B:
90%(v/v) AcN
10% (v/v) 20mM $CH_3COONa$
pH7.0

Gradient method:

| Segment | Minutes | Solvent A | Solvent B |
|---|---|---|---|
| 1 | 0-3 | 90% | 10% |
| 2 | 3-33 | 55% | 45% |
| 3 | 33-38 | 0% | 100% |
| 4 | 38-42 | 0% | 100% |
| 5 | 42-43 | 90% | 10% |

Flow rate: 1500µl.min$^{-1}$

| Detection: | initial | (at 22 min.) | (at 40 min.) |
|---|---|---|---|
| Excitation: | 230nm | 260nm | 230nm |
| Emmission: | 455nm | 313nm | 455nm |

Derivatization: double step.
Run time: 28 minutes.

Method 2b

This was an isocratic method similar to method 1b but also resolved alanine.

Solvent, detection and reagent as above (method 1b).

Run time: 35 minutes.

Method 3a

This gradient method gave better resolution of most peaks and allowed the detection of additional amino acids. OPA-derived amino acids were resolved using this method.

solvent A:

25mM $CH_3COONa$, pH5.7

4.5% (v/v) THF

3.0% (v/v) isopropyl alcohol (propan-2-ol)

solvent B:

Methanol

1.5% (v/v) THF

1.5% (v/v) isopropyl alcohol.

Gradient method:

| Segment | Minutes | Solvent A | Solvent B |
|---|---|---|---|
| 1 | 1-6 | 100% | 0% |
| 2 | 6-12 | 93% | 7% |
| 3 | 12-36 | 54% | 46% |
| 4 | 36-40 | 15% | 85% |
| 5 | 40-45 | 100% | 0% |

Flow rate: 1500µl.min$^{-1}$

Detection: Excitation: 230nm

Emmission: 455nm

Derivatization: double step; OPA-mercaptoethanol was used.

Run time: 42 minutes.

Method 3b

This isocratic method was for the separation of the internal standard from phenylalanine (ε-n-a-cap and phe, 0.5mM, used as standards).

| | |
|---|---|
| Solvent: | 25% (v/v) AcN in 50mM $CH_3COONa$, pH6.0. |
| Detection: | 340nm. |
| Derivatization: | single step, OPA-mercaptoethanol. |
| Flow rate: | 1000µl.min$^{-1}$ |
| Run time: | 42 minutes |

Method 3c

FMOC-derived secondary amino acids were resolved by this method which was run in parallel with methods 3a and 3b.

Solvents: A: as for method 3a
B: as for method 3a.

Gradient method:

| Segment | Minutes | Solvent A | Solvent B |
|---|---|---|---|
| 1 | 1-2 | 90% | 10% |
| 2 | 2-27 | 40% | 60% |
| 3 | 27-28 | 15% | 85% |
| 4 | 28-33 (reset) | 90% | 10% |
| 5 | 33-36 (equil.) | 90% | 10% |

| | | |
|---|---|---|
| Flow rate: | 1500µl.min$^{-1}$ | |
| Detection: | Excitation: | 260nm |
| | Emmission: | 313nm |
| Derivatization: | double step; OPA-mercaptoethanol | |
| Run time: | 30 minutes. | |

## 2.13 Organic Acid Estimation

Organic acids excreted by *S. coelicolor* into the medium during growth and actinorhodin production were measured by the following method.

### 2.13.1 Detection and measurement

Organic acids were analyzed by HPLC using a Philips PU4100 Liquid Chromatograph and additional equipment previously described for amino acid analyses (section 2.11.2.2). However, the spectrofluorimeter was replaced by a PYE UNICAM PU4021 Multichannel Detector. The column

used was a SHODEX IONPAK KC-811 column of 83mm internal diameter and 300mm length. This was packed with ion exchange resin gels (sulphonated rigid styrene-divinylbenzene copolymer). Separation of organic acids therefore occurred by a combination of ion exclusion and partition/adsorption. A SHODEX KC-810P column (6mm internal diameter, 50 mm length) was also used as a guard column for protection of the KC-811 column. Use of the autoinjector (Gilson Model 231) required initial rinsing of the needle with 75% (v/v) acetonitrile.

Separation and measurement of organic acids was carried out according to the manufacturers instructions. This involved an isocratic method using 0.1% (v/v) orthophosphoric acid as solvent at a flow rate of $0.5ml^{-1}.min.$ Detection of organic acids was by UV absorption at 210nm.

Identification of organic acid HPLC peaks was carried out using authentic standards (5mM or 10mM) of pyruvate, α-ketoglutarate, citrate, malate, fumarate, succinate and lactate. The samples analyzed were not diluted.

### 2.13.2 Positive identification of pyruvate and α-ketoglutarate

Positive identification of pyruvate was carried out by using the enzyme lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC1.1.1.27; from pig heart, Boehringer Mannheim). This catalyzes the reaction:

$$\text{pyruvate} + \text{NADH H}^{+} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}$$

The reaction was carried out by adding NADH to an aliquot of neat sample (100µl) to give a final concentration of NADH of 1.2 fold greater than the concentration of pyruvate in the sample (as measured by HPLC). This solution was then incubated with LDH (1 in 40 fold dilution) for 5 minutes at 37°C. The solution was then analyzed using the HPLC. The resulting lactate peak was verified using an authentic standard.

Additional identification of α-ketoglutarate was attempted by hydrolyzing aliquots of the culture samples with 1N HCl for 10 minutes in a boiling water bath. This was expected to result in the formation of succinate. Resulting solutions were then analyzed by HPLC.

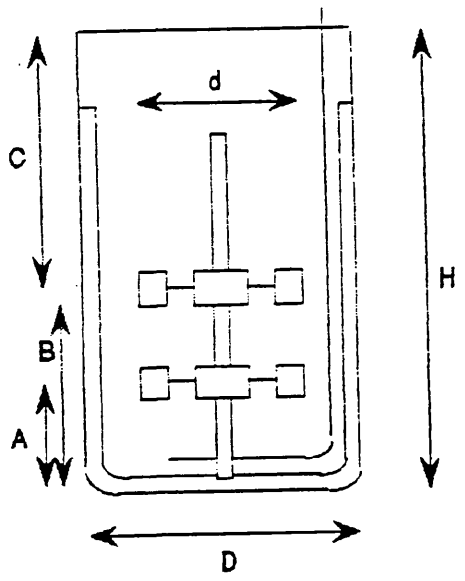



**Figure 2.1. Diagrammatic representation of the Bioengineering fermenter** (from Burke, 1991).

Key

| | | |
|---|---|---|
| H, height of fermenter | = | 51.7cm |
| D, diameter of fementer | = | 15.5cm |
| d, impellor diameter | = | 8.4cm |
| A, height to first impellor | = | 8.4cm |
| B, height to second impellor | = | 16.8cm |
| C, height from second impellor | = | 26.5cm |
| w, baffle width | = | 1.0cm |

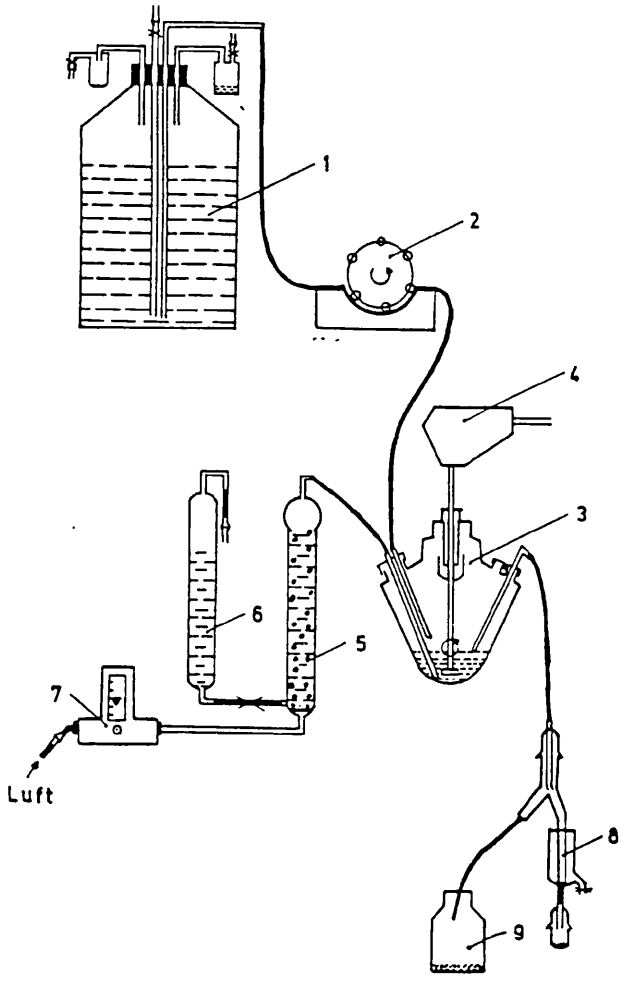



**Figure 2.2. Diagram of chemostat system used in Jena, FRG** (M. Roth, personal communication).

1, 10litre medium vessel.

2, peristaltic pump.

3, culture vessel (maintained at 30°C in a water bath).

4, Motor for glass stirrer; stirrer consists of one baffle.

5, Humidifier containing water through which air is passed.

6, Water reservoir for 5.

7, Reduction/regulation valve for air.

8, Collection vessel for samples.

9, Flask for effluent culture containing disinfectant.

# Chapter 3

# Development of cultivation methods for dispersed growth of *Streptomyces coelicolor* A3(2) in a defined minimal medium

## 3.1 Introduction

This chapter describes the methods used to obtain dispersed cultures of *S. coelicolor* which were suitable for the quantitative physiological studies detailed in chapter 4. Growth of *S. coelicolor* in a glucose-salts minimal medium and the associated problems are included. The following section contains a description of some existing methods used for producing dispersed growth of *Streptomyces*.

### 3.1.1 Methods for dispersed growth of *Streptomyces*.

As described in section 1.5.3, *Streptomyces*, although prokaryotic, have a mycelial growth pattern similar to that of the eukaryotic fungi and exhibit both pelleted and filamentous growth in liquid cultures (Williams *et al.*, 1974). The pelleted form usually pre-dominates. Because homogeneity is a prerequisite for physiological studies, it is detrimental to have these heterogeneous, pelleted cultures. Several methods have been used in an attempt to obtain dispersed streptomycete cultures. For example, mechanical means, *e.g.*, the use of stainless steel springs, glass beads or baffled flasks, have been augmented by the addition of specific chemicals to the medium. Hodgson (1982), while studying carbon catabolite repression in *S. coelicolor*, used a minimal medium containing PEG 6000 in addition to steel springs. Medium containing starch plus glass beads was also shown to promote dispersed growth of this species (Doull and Vining, 1989). The presence of PEG 6000 or starch would have increased the viscosity of the medium. This may have reduced the extent of aggregation of *S. coelicolor* mycelia, thus preventing pelleted growth.

Dispersed growth of another streptomycete species, *S. clavuligerus*, has been obtained in a fermenter containing a viscous, complex medium. Mycelial morphology was shown to be dependent on the speed of the stirrer in the fermenter (Belmar-Beiny and Thomas, 1991). Increasing the speed of the stirrer to 1300rpm accelerated the initial phase of fragmentation, during which long, highly branched mycelia were observed to be sheared into shorter, less branched fragments. The growth yield of *S. clavuligerus* and production of clavulanic acid were not affected by the stirrer speed. This was probably due to the small lengths (several mm) and diameters (1μm to 5μm) of the mycelia. Fungi also exhibit dependency on rate of agitation for

both growth and productivity (Bader, 1986), possibly because their hyphae achieve lengths of 50mm to 100mm and diameters 10μm to 20μm.

Growth of certain fungi in the presence of high molecular weight anions resulted in the formation of loose mycelial clusters (Elmayergi *et al.*, 1973; Trinci, 1983). This may have been due to electrostatic repulsion occurring among germinating spores which were coated with the polymer. The resulting loose pellets exhibited enhanced growth rates, glucose utilization and $CO_2$ evolution, possibly a result of an increase in the interfacial surface area. The electrophoretic mobilities of certain fungi were also observed to alter in the presence of junlon-110, a cross-linked polyacrylate. *Aspergillus niger* and *Phanerochaete chrysosporium* exhibit aggregation during growth in submerged culture. However, when coated with junlon-110, both species showed peaks of negative mobility at pH4.0 (Jones *et al.*, 1988). This was similar to the peak shown in pH mobility curves of *Geotrichum candidium*, a non-aggregating species. In the absence of junlon-110, the aggregating strains had shown very dissimilar curves.

To examine the effect of electrostatic repulsion between streptomycete mycelia, comparisons of the growth of *S. lividans* and *S. coelicolor* were carried out in the presence of three negatively charged polymers: Carbopol (a carboxypolymethylene), junlon-110 and agar (a polysaccharide; Hobbs *et al.*, 1989). The effect of an uncharged polymer, PEG 6000, was also studied. Supplementation of the medium with the charged polymers resulted in the most dispersed growth, with Carbopol and junlon-110 giving the best results. Junlon-110 was chosen for physiological studies by Hobbs *et al.* (1989) because it was more easily suspended in the growth medium.

Methods of growth of *Streptomyces* can also affect the extent of pelleting or dispersal in cultures. As with other prokaryotes, the most common method for growing *Streptomyces* is batch cultivation (section 1.5.1). However, since μ is the maximum for the conditions, growth of *Streptomyces* in batch culture is conducive to a high degree of branching leading to entanglement and pellet formation. Alternatively, continuous cultivation allows μ to be controlled at very low dilution rates, thus preventing the formation of a large number of branches. Highly dispersed growth, almost filamentous, which results from growth of unbranched mycelia or mycelia with very few branches, has been observed in prolonged continuous cultivation of *S. lividans* (D. Noack, personal communication). In addition, *S. tendae* mycelia have been shown to undergo a change in morphology when

switched from batch to continuous culture. Pelleted biomass was reduced in quantity and was maintained thereafter as mycelial mats (Lohr *et al.*, 1989). Such a change in morphology has also been observed in continuous cultures of *S. thermonitrificans* (Burke, 1991). Kretschmer (1981; cited by Lohr *et al.*, 1989) stated that continuous cultivation of *Streptomyces* was possible only if the mycelia were disrupted by shear forces. New growing points would then be formed from old hyphal regions, thus counteracting aging (Riesenberg and Bergter, 1979; cited by Belmar-Beiny and Thomas, 1991). This would not occur with pelleted cultures.

As mentioned previously, dispersed growth of *Streptomyces* can be obtained by a variety of mechanical and chemical means. During dispersed growth, hyphal tips undergo greater exposure to the medium and do not endure the nutrient limitations common to pellets. Although increased dispersion of *S. clavuligerus* does not affect clavulanic acid production (Belmar-Beiny and Thomas, 1991), the presence of junlon or starch in cultures of *S. coelicolor* increased the production of actinorhodin (Hobbs *et al.*, 1989; Doull and Vining, 1989), suggesting that, in this case, morphology may affect productivity.

## 3.2 Objectives

The aims of this chapter are as follows:

i) to describe the methods for batch cultivation of *S. coelicolor*. The previous section described the use of high molecular weight polymers. As described in section 1.5.3, growth of *Streptomyces* in minimal media can lead to pellet formation which is unsuitable for physiological studies. Experiments with *S. coelicolor* involving the addition of high molecular weight anions to the medium, which cause electrostatic repulsion between spores and mycelia, have resulted in increased dispersion. The presence of neutral high molecular weight compounds has also aided in dispersal, possibly by increasing the viscosity of the medium. The use of such chemical additives to obtain dispersed growth of *Streptomyces* therefore appears advantageous, especially for physiological studies requiring growth in minimal medium. However, these compounds are all carbon based. The calculation of metabolic fluxes requires a balance of carbon where input is equivalent to output (Holms, 1986; section 1.10). Additional carbon compounds in the growth media may interfere with this balance. For

example, junlon-110 is extremely difficult to remove from microbial biomass (Trinci, 1983) and, therefore, hampers accurate determination of carbon content of the cells. Consequently, it was necessary to examine alternative methods by which *S. coelicolor* could be grown in a dispersed manner in a defined minimal medium. This involved the use of mechanical means. Section 3.3 describes these methods and their effect on biomass yield and antibiotic production.

ii) to examine the growth of *S. coelicolor* in a chemostat. Growth in batch culture occurs at a maximum growth rate for the conditions (described in section 1.5.3). This is conducive to the formation of a high number of branches, thus promoting entanglement of hyphae and pellet formation. Growth rate may be controlled in a chemostat. Thus pellet formation may also be controlled and possibly prevented. It would have been of interest to examine mycelial formation by *S. coelicolor* at low growth rates and to determine the effect of changes in dilution rate on mycelial morphology in the chemostat.

iii) to determine the extent of actinorhodin production during continuous cultivation. Production of actinorhodin has been observed to be dependent on phosphate concentration (Hobbs *et al.*, 1990) and possibly on nitrogen concentration (Doull and Vining, 1990). Use of the chemostat with nutrient-limiting media would determine to which extent each nutrient was involved in regulation of production of actinorhodin. In addition, although actinorhodin is reported to be produced in stationary phase (*e.g.* Hobbs *et al.*, 1990), transcripts from the *actIII* gene have been detected during the transient period between growth and stationary phases (Strauch *et al.*, 1991). Growth rate decreases during this period. It was therefore possible that synthesis of the antibiotic was associated with very low growth rates. These growth rates may be obtainable in the chemostat resulting in simultaneous production of biomass and actinorhodin.

## 3.3 Batch cultivation of *S. coelicolor* 209 in flasks.

Conditions for the batch cultivation of *S. coelicolor* in HMM (section 2.5.1), which includes junlon, had been defined previously (A. Moran, unpublished results). They were also based on those described by Hobbs *et al.* (1989). The conditions allowed production of the red and blue antibiotics, undecylprodigiosin and actinorhodin. These antibiotics were produced

towards the end of the growth phase and during stationary phase respectively. Growth and productivity differed slightly between cultures, but were, in general, reproducible.

The studies described in chapter 4 involved the determination of carbon in mycelial cultures, *i.e.*, in both the biomass and the medium. As discovered by Trinci (1983), it was not possible to remove junlon completely from fungal biomass because of binding of the polyacrylate to the surface of the mycelia (Jones *et al.*, 1988). This resulted in an immeasurable over estimation of the carbon content of the biomass, thus rendering the use of junlon unsuitable. There was, therefore, a requirement for an alternative means of dispersion.

### 3.3.1 Comparisons of methods for dispersed growth.

Spores of *S. coelicolor* 209 were pre-germinated following the method of Hodgson (1982; section 2.4.3.1). This ensured synchronous germination of the spores. Pre-germinated spores were inoculated into 50ml of NMM-J (HMM without junlon; section 2.5.1) in 250ml shake flasks using an inoculum concentration to give a final value of $5x10^5$ spores.ml$^{-1}$ of culture. Growth under various conditions, using different dispersal techniques, led to the following observations concerning dispersal and actinorhodin production. The experiments were performed in duplicate.

In the folllowing observations, descriptions of the mycelial morphology are given. They are defined as: pelleted, compact spheres with only a few individual hyphae at the periphery; semi-dispersed, small aggregates with less dense centres and some individual hyphae visible at the centre and at the periphery; dispersed, large areas of individual hyphae observable as a mycelial mat.

i) Conical flask alone. Cultures grown for 48 hours showed differing sizes of very compact pellets which had produced small quantities of undecylprodigiosin, as indicated by a light red coloration.

ii) Flask with stainless steel spring coiled around the base. Cultivation for 45 hours resulted in a few large pellets with the majority of mycelia growing on the wall of the flask at the liquid-air interface. This was a disadvantage as mycelia growing on the wall would have utilized, and

therefore removed, nutrients from the medium but were no longer part of the liquid culture. It also led to a heterogeneous population of cells. In an attempt to prevent cell adhesion, the glass was coated with repelcote (dichlorodimethylsilane; Hopkins and Williams, England) . This, however, did not reduce the extent of mycelial growth on the flask walls. No antibiotic production was observed.

iii) Flask containing 22 glass beads, of diameter 0.2cm to 0.3cm (approximately 1g). Initial cultures, with a final spore concentration of $5x10^5$ spores.ml$^{-1}$ of culture, demonstrated semi-dispersed growth and, after 48 hours incubation, the cultures were light purple in colour indicating simultaneous production of undecylprodigiosin and actinorhodin. After a further 24 hours the mycelia had undergone additional dispersion and showed increased production of actinorhodin. Four additional experiments with glass beads were carried out with $1x10^6$ spores.ml$^{-1}$ of culture. These cultivations gave similar results with respect to production of actinorhodin, *i.e.* longer cultivations resulted in a deep blue colouration which is indicative of the sole production of actinorhodin. However, dispersion of these cultures, in comparison to those with $5x10^5$ spores.ml$^{-1}$ of culture, was greater and looser pellets were formed. This was possibly due to the higher inoculum concentration.

Two subsequent incubations of *S. coelicolor* with glass beads resulted in no observable mycelial growth. This may have been a consequence of the addition of the glass beads to the cultures at the time of inoculation. It is possible that the beads may have sheared the vulnerable germ-tubes from the spores. A further experiment demonstrated that the problem was alleviated by the addition of the glass beads to the cultures 24 hours after inoculation.

iv) Baffled flasks. Baffles were formed inside the flasks by pushing the glass in from the outside at three equidistant places. Mycelial growth in these flasks was semi-dispersed but there was infrequent production of actinorhodin. In most cases, undecylprodigiosin was observable after 50 hours of incubation. Comparisons of growth from spore concentrations ranging from $5x10^3$ spores.ml$^{-1}$ of culture to $5x10^6$ spores.ml$^{-1}$ of culture were made in an additional experiment. Greater dispersion was observed with a higher concentration of spores. This supported the results observed in part (iii) of this section. Undecylprodigiosin production was observed only in cultures inoculated with low concentrations of spores.

A disadvantage encountered during the use of baffled flasks was the smaller internal volume of the flasks. Loss of mycelia occurred because of the tendency of the liquid to splash onto the cotton wool bung. This may also have caused a reduction in the extent of diffusion of oxygen into the flasks.

v) Baffled flasks plus stainless steel springs; the springs were coiled underneath the baffles. In this case, the observations were a combination of those observed in parts (ii) and (iv) of this section: in addition to mycelial wall growth in the presence of repelcote, splashing occurred onto the cotton wool bung. Pellets remaining in the medium were very small and tight with no observable antibiotic production.

vi) Baffled flasks plus glass beads. Glass beads, the same number as used in part (iii), were added to the flasks at the time of inoculation. Growth under this condition was pelleted with no antibiotic production. A disadvantage of this system was the presence of foam at the top of the medium, possibly due to lysis of the mycelia caused by the extra shearing forces from both the baffles and the beads. A possible explanation for pellet formation was lysis. This would have resulted in less colony forming units, the hyphae of which would have undergone a high rate of branching in the nutritious environment.

From these experiments, the most favourable method for dispersed growth and actinorhodin production was observed to be the addition of glass beads to a culture in a normal shake flask, 24 hours after inoculation. This cultivation method was demonstrated to be reproducible by five additional growth experiments.

However, dispersal was not comparable to that observed in the presence of junlon. Figure 3.1 demonstrates the extent of dispersion of mycelial pellets grown in HMM and in NMM-J. In the presence of junlon (in HMM), hyphae were visible at the periphery and in the centre of "aggregates" formed during growth (figure 3.1a). Therefore the mycelia were very dispersed and were exposed to the medium. Pelleted growth was usually observed in NMM-J without dispersing agents (figure 3.1b). The pellets were very large with extremely dense centres where production of undecylprodigiosin occurred. However, the addition of glass beads acted as a shearing agent and promoted greater dispersion, to a certain extent (figure 3.1c). Aggregation of the mycelia still occurred, but individual hyphae could be seen at the periphery and at the centres of the small pellets. Although

dispersion with glass beads was not to the extent of that with junlon, in comparison to alternative mechanical means, the beads exhibited the best degree of dispersion.

Determination of glucose utilization (by the GOD PERID method, section 2.7.4.1) in cultures grown from $5x10^5$ spores.ml$^{-1}$ of culture in the presence of glass beads or junlon, revealed that the cells coated with junlon exhibited a faster rate of glucose uptake (figure 3.2). Although the time interval between samples is large, the graph clearly illustrates the complete utilization of glucose from HMM by 69.5 hours after inoculation. The cultures grown in NMM-J with glass beads had only utilized 0.6g.l$^{-1}$ of glucose by this time. The difference in glucose uptake possibly reflects the extent of dispersion of the mycelial pellets. Because junlon causes electrostatic repulsion between mycelia, it may have been possible for nutrients in the medium to diffuse to central regions of the aggregates. Such diffusion was probably prevented in pellets grown in the presence of glass beads because of high mycelial density.

To determine if the number of mycelial aggregates was responsible for the slow utilization of glucose in cultures containing glass beads, the numbers of colony forming units (CFUs; section 2.4.4) derived from cultures including junlon or glass beads were compared. Hobbs *et al.* (1989) had observed a higher number of CFUs formed in the presence of junlon. It would have been interesting, therefore, to see if tight pellet formation and possibly mycelial death, caused by glass beads shearing growing apical hyphae, had contributed to a reduction in this number. The number of CFUs produced by inoculating Emmersons agar plates with appropriately diluted culture samples (section 2.4.4) was, however, similar to those obtained from the culture containing junlon:

glass beads: $9.5x10^4$ cfu's.ml$^{-1}$, $1.08x10^5$ cfu's.ml$^{-1}$
junlon: $7.7x10^4$ cfu's.ml$^{-1}$, $1.82x10^5$ cfu's.ml$^{-1}$.

The slow uptake of glucose in cultures containing glass beads was therefore not attributable to the presence of fewer mycelial aggregates. Glucose was also shown not to be a limiting factor involved in the onset of antibiotic production in cultures with glass beads. The cultures grown in medium including junlon or glass beads both exhibited a long lag phase prior to glucose utilization and actinorhodin was observed to be produced at similar times (54 hours), indicating the simultaneous onset of stationary phase.

### 3.3.2 Macromolecular composition of *S. coelicolor* 209

The following section describes a comparative study of the determined macromolecular composition of *S. coelicolor* 209 determined from mycelia harvested during different stages of growth, as indicated by production of undecylprodigiosin and actinorhodin. Cultures growing under the same conditions would be expected to contain similar proportions of macromolecules (RNA, ribonucleotide; DNA, deoxyribonucleotide; protein) at the same stage of growth. The study was also useful for later determinations of macromolecular composition by predicting whether antibiotic production was a good parameter to use as an indication that cultures were at the same stage of growth. Therefore, biomass samples from single cultures and from cultures pooled at similar extents of antibiotic production were assayed for macromolecular content. An estimation of carbohydrate content was also included. The macro-molecular components were separated by a fractionation method (section 2.9.1; described fully in section 4.1.2) and measured by spectrophotometric assays (section 2.10).

Table 3.1 gives the macromolecular compositions of biomass from eight sets of cultures grown in NMM-J (a set is one or two cultures) and harvested at times prior to and during antibiotic production. Samples A, B, G and H were obtained by harvesting biomass from individual cultures. However, samples C, D, E and F resulted from pooling cultures of similar coloration, *i.e.* at similar stages of antibiotic production. Biomass at similar stages of undecylprodigiosin production (samples A to E) showed an overall pattern of differing macromolecular content. Allowing a 10% error range, samples A and B revealed similarities in their content. However, samples C and D had very variable RNA values which reduced their overall similarity to each other. Pooling of the cultures did not result in the expected "averaging-out" of differences.

A similarity in carbohydrate values was shown by samples E and F, both of which had produced actinorhodin. RNA values were also comparable, although DNA contents and protein contents were dissimilar. This is in contrast to those samples producing undecylprodigiosin which showed relatively similar contents of DNA and protein. Comparison of samples E and F with samples A, B, C and D revealed slight similarities in the contents of both sets of samples. Samples G and H were harvested from cultures lacking in antibiotic production. The overall contents were, however, generally comparable with those of samples C, D, E and F.

These determinations suggest that antibiotic production is not a good indication of cultures in similar physiological states. Dissimilarities in macromolecular content existed between cultures producing the same antibiotic, while similarities were present in cultures producing different secondary metabolites. Discrepancies in macromolecular content were not removed by the pooling of cultures. Small differences were also present between samples harvested at the same time, irrespective of antibiotic production. This indicates a general variation in growth between biomass cultivated under the same conditions in different flasks. Complementary experiments were also carried out with *S. coelicolor* 1147 to determine the extent of variation in cultures of this strain (section 4.4.1).

## 3.4 Batch cultivation of *S. coelicolor* 1147 in flasks

Subsequent cultivations with *S. coelicolor* 209 spores resulted in the production of insufficient quantities of biomass for analysis, necessitating the frequent pooling of cultures. According to collaborators at UMIST (G. Hobbs, personal communication), *S. coelicolor* 1147 exhibited a faster and more stable pattern of growth and actinorhodin production than *S. coelicolor* 209. Both strains of *S. coelicolor* contain the plasmid SCP1, although it is integrated into the chromosome of *S. coelicolor* 209. The presence of SCP1 and SCP2 free in the cytoplasm may contribute to the better growth characteristics of *S. coelicolor* 1147. SCP1 encodes the biosynthetic pathway for the antibiotic methylenomycin, but production is negligible in NMM (and therefore NMM-J) with sodium nitrate as the nitrogen source (Hobbs *et al.*, 1992).

Section 2.4.3.2 describes the methodology used for cultivation of *S. coelicolor* 1147. Pre-germination of the spores was not carried out. Pre-germination of *S. coelicolor* 209 had resulted in a loss of 90% to 93% of the viable spore concentration, as measured by the number of colonies formed from the growth of appropriately diluted spore suspensions. Fresh spores of *S. coelicolor* 1147 were used as inocula and spore concentrations of $1x10^7$ spores.$ml^{-1}$ of culture resulted in semi-dispersed to dispersed growth. A high inoculum concentration, in addition to aiding dispersion, increased the number of spores likely to germinate at a similar time.
The onset of production of actinorhodin was observed to be reproducible within 48 to 60 hours after inoculation when the residual glucose concentration (3.6g.$l^{-1}$, as measured by the GOD PERID assay; section 2.7.4.1)

was greater than half the initial concentration at this time (figure 3.3). This observation was similar to that in section 3.3.1 concerning *S. coelicolor* 209 and slow glucose uptake. Determination of phosphate concentration in the supernatants of the *S. coelicolor* 1147 cultures (section 2.10.5) revealed a drop in concentration from $1.5g.l^{-1}$ to approximately $1.3g.l^{-1}$. The latter level was reached at the time of onset of actinorhodin production. Previous experiments carried out to determine a suitable composition of medium for growth of *S. coelicolor* 209 in the presence of junlon had indicated that a phosphate concentration of $1.3g.l^{-1}$ was important for actinorhodin production (A. Moran, unpublished results). Concentrations of phosphate, used at an initial range of $0g.l^{-1}$ to $3.0g.l^{-1}$ $KH_2PO_4$, were determined to be $1.3g.l^{-1}$ at the onset of actinorhodin production in those cultures which synthesized the antibiotic. Complete utilization of phosphate was obviously not necessary for the switch into actinorhodin production. However, the switch itself may have been an indication of reducing phosphate concentrations, *i.e.*, it may have been a sensor of inorganic phosphate levels.

Morphological observations were also made on the flask cultivations of *S. coelicolor* 1147 (figure 3.4). Analogous with the growth of *S. coelicolor* 209 in HMM and in NMM-J with and without glass beads (section 3.3.1), the differences in morphology were striking. The mycelia grew in an extremely dispersed fashion in the presence of junlon (figure 3.4a), whereas very large, dense pellets were formed in NMM-J (figure 3.4b). Smaller pellets were observed in NMM-J in the presence of glass beads. However, they had a battered appearance and individual hyphae could be seen in the centres of the pellets (figure 3.4c).

*S. coelicolor* 1147 exhibited better and more reproducible production of actinorhodin than *S. coelicolor* 209, but procurement of only small amounts of biomass was still a source of problems. To obtain a curve describing batch growth of *S. coelicolor* 1147 in shake flasks, it was necessary to pool cultures. Pooling provided enough biomass to measure (the dense mycelia of *S. coelicolor* was not bouyant enough for optical density measurements), but undoubtedly led to a degree of heterogeneity. An outline growth curve of *S. coelicolor* 1147 was obtained using ten flask cultures. Rough indications of glucose utilization, and biomass and actinorhodin production were obtained (figure 3.5). Three cultures were used for each of the first two sets of data (points) on the graph, while the second two sets were each obtained from two cultures. However, the experiment was not repeated with flask

cultures because of the heterogeneity of samples. Growth curves obtained from fermenter samples are given in the following section (3.5.1).

Scale-up of volumes from 50ml cultures to 100ml and 400ml cultures was attempted to obtain greater amounts of biomass. However, shaking of cultures of 100ml in 500ml flasks with 2g of glass beads led to breakage of the flasks, and 400ml cultures in 2litre flasks were pelleted because it was difficult to scale-up the quantity of glass beads required for this volume. Sufficient quantities of biomass were therefore sought in batch cultivations carried out in the fermenter.

## 3.5 Batch cultivation of *S. coelicolor* 1147 in fermenters

### 3.5.1 The Bioengineering fermenter

To obtain biomass samples which were sufficient in weight for the numerous analyses described in chapter 4, growth of *S. coelicolor* 1147 was carried out in a Bioengineering fermenter. The fermenter was of 8L volume with a working volume of 7litres. This was suitable for removal of large sample volumes. Descriptions of the fermenter and associated equipment are given in section 2.6.2. Included in this section are the methods used for inoculation and sampling.

Fermentations were run at 30°C. Depending on the time of year, this temperature ranged from 5°C to 12°C above room temperature. As differences in the extent of the saturation of the air with water were inevitable, evaporation was possible . To determine the necessity for cold water to run through the condenser (part of the air outlet system), measurements of the Tris (tris (hydroxymethyl) amino ethane) content of glucose-free NMM-J were taken over a time period estimated to be equivalent to that of an *S. coelicolor* fermentation. An increase in carbon concentration of the medium indicated an increase in Tris concentration, resulting from evaporation. Tris (MW 121.1) was used at a concentration of 10mM, measured as 480ppm of carbon (as determined by the TOCA; section 2.7.3). Table 3.2 shows the increase in concentration of Tris in the medium. By 95 hours this had increased by 15%. The condenser was therefore used throughout fermentations.

Eleven batch fermentations were carried out with *S. coelicolor* 1147 in the Bioengineering fermenter. Biomass samples harvested from these fermentations at different time points were also analyzed for macromolecular content. Large sample volumes were necessary for sufficient biomass for the macromolecular composition analyses described in chapter 4. These volumes were of 1litre up to 50 hours after inoculation and 500ml thereafter. In addition, for cultures to be maintained at a constant temperature, the temperature probe in the fermenter had to be submerged. This required a minimum volume of 3litres in the fermenter, allowing only 4litres of culture to be removed prior to the final sample. Therefore, only small numbers of samples could be taken and it was not possible to take them in triplicate.

Initial experiments with an aeration rate of $1v.v^{-1}.min^{-1}$ and an agitation rate of 400 or 600rpm, had resulted in mycelial morphology comparable to that in flasks, *i.e.*, the majority of the mycelial aggregates were semi-dispersed. However, increasing the agitation rate to 700rpm led to the formation of looser aggregates. Preliminary fermentations also revealed the disadvantageous tendency of *S. coelicolor* mycelia to grow on the vessel walls at the air-liquid interface. This was observed from approximately 12 hours after inoculation. Addition of spores into the fermenter while aeration and agitation were at their maximum rates resulted in incomplete mixing of the spores within the medium. Air pressure then caused the spores to be splashed onto the vessel wall. This was prevented by interrupting aeration and agitation at the time of inoculation which allowed the spores to be carried into the vortex (caused by resuming agitation). Thus, the extent of mixing was increased and that of wall growth was decreased.

Five fermentations (**a** to **e**; figure 3.6) were used to examine the pattern of growth of *S. coelicolor* in batch culture. Fermentations **a**, **b**, **c**, and **e** were performed under the same conditions. Extra glucose was added to fermentation **d** at 44h after inoculation. Growth in the fermenter followed a pattern similar to that observed in flasks (section 3.4): the end of growth phase was indicated by the production of undecylprodigiosin (as determined visually) and stationary phase (as measured by the cessation of increase in wet weight, approximately 40 to 60 hours after inoculation) was generally reached prior to the complete utilisation of glucose. Production of actinorhodin was observed prior to a reduction in the rate of biomass formation in some cases. In general, as described below, the pattern of

biomass production and actinorhodin synthesis was variable between fermentations.

Cultivation of *S. coelicolor* 1147 in fermentation **a** resulted in a maximum biomass concentration of 28g wet weight.$l^{-1}$, but this decayed while glucose continued to be utilized. A similar decrease in concentration of actinorhodin was also observed. The reduction in biomass concentration signified the occurrence of lysis but the breakdown of actinorhodin was unusual as it was not observed in other fermentations. However, glucose was utilized at a high rate during this fermentation and breakdown of actinorhodin occurred in the absence of the carbon source. The antibiotic was produced at a much slower rate during fermentation **b**, but the end value was comparable to the maximum value in fermentation **a**. A difference in biomass production was observed, however, with a maximum concentration of 6g.$l^{-1}$, although this maximum was reached at a time similar to that in the initial fermentation. Lysis followed sharply to give less than 1g.$l^{-1}$ biomass at a glucose concentration of 1g.$l^{-1}$. The higher ratio of actinorhodin to biomass may have been due to the concentration of residual carbon source at the end of growth which was possibly directed towards secondary metabolite production. Lytic products may also have been sources of carbon.

Growth during fermentation **c** was different to both previous fermentations. Very little lysis occurred and a period resembling stationary phase was observed. The maximum concentration of biomass was 12g.$l^{-1}$ but actinorhodin, which reached concentrations comparable to previous determinations, was produced simultaneously with biomass. This suggests the culture was physiologically heterogeneous with a minority of cells growing towards stationary phase very quickly possibly because of the formation of mycelial pellets. These "old" cells would have produced the actinorhodin measured while the remaining cells continued to grow. The final value of actinorhodin concentration in the culture was not measured because of interference of lytic products in the optical density measurements.

Biomass production in fermentation **d** was also very low (6g.$l^{-1}$) and lysis occurred but over an extended period of time. Synthesis of actinorhodin was observed to be delayed in this fermentation until extra glucose was added to the culture (80h). However,the concentration of antibiotic only reached a third of the values in the other fermentations. This may have been

attributable to the low number of viable cells at the time of addition of glucose.

Dry weight measurements (filtration method; section 2.7.2.1) were taken during fermentation **d.** Only three samples had been removed for chemical analysis and, therefore, wet weight measurements. A rough approximation of stationary phase was obtained using these measurements which occurred at a glucose concentration of $3g.l^{-1}$ (analogous with fermentations **b, c** and **d**). The final dry weight biomass concentration was extremely low, giving a yield of $0.25g.g^{-1}$ of glucose; cultivation of *S. thermonitrificans* in semi-defined medium in the same fermenter had resulted in a yield of $0.78g.g^{-1}$ of glucose. Production of actinorhodin occurred during stationary phase in fermentation **d** to a maximum concentration of greater than $30mg.l^{-1}$.

Cultivation of *S. coelicolor* in the Bioengineering fermenter therefore resulted in variable results. Similarities included the time of onset of stationary phase and/or lysis and the final concentration of actinorhodin. However, this concentration was reached irrespective of biomass concentration. Dissimilarities observed in the growth and antibiotic production of *S. coelicolor* in shake flasks were obviously repeated in the fermenter.

Morphological observations were made throughout fermentations. These observations suggested that a change in morphology may have occurred when actinorhodin was produced, *i.e.*, from semi-dispersed to looser pellets. The pellets appeared to be floccular and, at later stages in the fermentation, formed mycelial mats. This possible morphological change may have been reflected in biomass weight measurements. Comparison of wet weight and dry weight values (as measured by filtration, section 2.7.2.1) revealed that as a fermentation progressed, the proportion of dry mycelial weight per gram of wet weight increased (table 3.3; right-hand columns). Additional dry weight estimations were calculated using the carbon content of the wet weight biomass (assuming 1g dry weight biomass was composed of 50% carbon, section 2.7.3). These estimations were found to be similar to the measured values, within 10% error (table 3.3; left-hand columns).

Additional growth curves were obtained using the dry weight measurements (figure 3.7). On comparison with the pattern of growth in fermentations **a** to **c** in figure 3.6, differences were found in each fermentation. In fermentations **a** and **b**, no lysis was indicated with dry

weight measurements and the time of onset of stationary phase in fermentation **a** was later. The growth pattern in fermentation **c** (figure 3.7) was irregular, suggesting that dry weight measurements may have been inaccurate (although standard errors were very small). Measurement of dry weight by filtration was therefore found to be not suitable for all samples, especially those harvested early in a fermentation. Measurements of some samples gave negative values. In addition, high standard deviations were obtained with this method. Alternative methods of dry weight determination, such as the use of foil cups or corex tubes (section 2.7.2) were used but high standard deviations were also obtained. Therefore, subsequent dry weight determinations were estimated by measurement of carbon in the biomass (section 2.7.3).

An attempt was made to reduce the differences observed between fermentations. Spores were used from a single stock spore suspension stored in 20% (v/v) glycerol at -20°C. However, inoculation with these spores resulted in a high incidence of wall growth and little or no actinorhodin production, but mycelial aggregates were dispersed. Reversion to inoculation of the fermenter with fresh spores resulted in better production of actinorhodin.

### 3.5.2 Batch cultivation in Jena

Batch fermentations were performed in Jena (Germany) with the aims of improving dispersion, increasing biomass yields and obtaining reproducible production of actinorhodin by *S. coelicolor* 1147. Because the production of actinorhodin occurred in the presence of excess carbon, phosphate (see section 3.4) and nitrogen (Hobbs *et al.*, 1990; Doull and Vining, 1990), additional experiments were carried out in an attempt to identify potential factors involved in the switch from primary to secondary metabolism.

Cultivations were performed in a fermenter with a working volume of 3litres. Included in the vessel were probes for the measurement of temperature, pH and dissolved $O_2$ concentration (DO). $CO_2$ was also measured in the effluent gas. All measurements were traced on a recorder. Microscopic observations during a fermentation revealed the tendency of young mycelia to aggregate and form pellets. However, individual hyphae were visible within some pellets and some fragmentation had occurred, as detected by the appearance of long hyphae with few branches. Actinorhodin

was observable in the medium approximately 48 hours after inoculation (figure 3.8).

During the growth phase of a preliminary fermentation, the DO level had fallen to below 40% of the initial concentration (figure 3.9a). A similar reduction had also been observed prior to production of undecylprodigiosin (figure 3.9b). Therefore, the hypothesis was made that $O_2$ was a potential limiting factor involved in regulation of production of secondary metabolites. This hypothesis was subsequently rejected because, in successive experiments, DO levels did not fall below 75% although actinorhodin was produced. Investigation into this matter revealed the occurrence of a mechanical fault during the first fermentation, which had caused a reduction in air flow. However, a pattern in both DO and pH measurements was observed in the initial experiment whereby the parameters increased simultaneously prior to a large increase in actinorhodin production (figure 3.9c). This had also been seen before the onset of undecylprodigiosin production (figure3.9b). The synchronous increase in two or more variables in a fermentation had been previously suggested to indicate the time of switching into secondary metabolism by fungi (H.H. Große, personal communication).

## 3.6 Effect of phosphate on actinorhodin production

Actinorhodin was observed to be produced at phosphate concentrations of approximately $1.3g.l^{-1}$ in shake flask cultures of *S. coelicolor* 1147 grown in NMM-J (section 3.4). It was assumed that production of the antibiotic also occurred in the presence of excess phosphate in fermenter cultures. However, maximum concentrations of actinorhodin reached approximately $30mg.l^{-1}$, only a quarter of that measured in cultures grown in HMM ($120mg.l^{-1}$; Hobbs *et al.*, 1989). In an attempt to increase the extent of actinorhodin production, a small, preliminary experiment was performed in which the effects of differing concentrations of phosphate on actinorhodin and biomass production were studied. *S. coelicolor* 1147 was grown in NMM-J in shake flasks including $1.5g.l^{-1}$ $K_2HPO_4$ and $2.0g.l^{-1}$ $K_2HPO_4$. Production of both actinorhodin and biomass was observed to be increased in the culture containing $2.0g.l^{-1}$ $K_2HPO_4$, but a striking difference between the two cultures was found on expressing actinorhodin in terms of biomass weight ($mg.g^{-1}$ of dry weight biomass; table 3.4). Actinorhodin production was shown to be over 2 fold greater in the culture containing the

higher phosphate concentration. A repetition of the experiment gave a similar result. A further preliminary experiment also showed that 2.0g.l$^{-1}$ phosphate was optimal for production of actinorhodin. Growth and actinorhodin concentration were determined in cultures containing 1.5g.l$^{-1}$, 2.0g.l$^{-1}$, 2.5g.l$^{-1}$, 3.0g.l$^{-1}$, 3.5g.l$^{-1}$ and 4.0g.l$^{-1}$ $K_2HPO_4$. Final concentrations of actinorhodin in the culture containing an intial concentration of 3.0g.l$^{-1}$ $K_2HPO_4$ and greater were measured at less than the concentration determined in the culture with 2.0g.l$^{-1}$ $K_2HPO_4$ (table 3.5.)

## 3.7 Continuous cultivation of *S. coelicolor* 1147

This section describes experiments in a continuous culture system performed in Jena on the growth of *S. coelicolor* 1147 and production of actinorhodin. A description of the chemostat is given in section 2.6.2. Modifications to the medium and pre-cultivation techniques are included below. Samples were taken on an 8 hourly basis throughout the fermentations and were assayed for residual glucose concentration, pH and production of actinorhodin (see section 3.7.5).

### 3.7.1 Medium Modifications

Preliminary continuous culture experiments were carried out in Glasgow using NMM-J. This resulted in a carbon-limited culture. Residual glucose concentrations during steady-state were measured (using the GOD-PERID reagent; section 2.7.4.1) to be 0.05 to 0.08g.l$^{-1}$ (0.32 to 0.46mM), which may reflect the affinity of the glucose uptake system in *S. coelicolor*. No observable production of actinorhodin occurred at any time during this continuous culture. Actinorhodin is produced during the stationary phase of batch cultures of *S.coelicolor* 1147 (Hobbs *et al.*, 1989), although production was occasionally observed prior to this phase (sections 3.4 and 3.5.1). Therefore, because of the nature of continuous culture, production of the antibiotic was not expected.

Sections 3.4 and 3.5.1 describe the production of actinorhodin in batch cultures prior to the complete utilization of glucose. The available phosphate concentration was also high, at 1.3g.l$^{-1}$. With respect to the initial concentration of glucose, 4g.l$^{-1}$, a maximum biomass yield of less than 25% (*i.e.*, 1g dry weight biomass) was obtained, perhaps indicating a very low

growth rate. Hobbs *et al.* (1990) showed that high concentrations of phosphate inhibited actinorhodin production (also shown by Doull and Vining, 1990), with maximal production occurring at 0.38mM phosphate in batch culture. One aim of the experiment was to discover if actinorhodin could be produced in continuous culture, perhaps at a low dilution rate and at a low phosphate concentration. A phosphate-limited medium, based on NMM-J, was therefore developed.

Table 3.6a shows the required concentrations of glucose, nitrogen and phosphate for the continuous cultivation of *S. lividans* (M. Roth, personal communication). Replacement of nitrate for ammonium and $K_2HPO_4.3H_2O$ for $KH_2PO_4$ resulted in a recalculation of concentrations which were then increased by a factor of 4 for sufficient production of *S. coelicolor* biomass (table 3.6b). A preliminary cultivation with $6g.l^{-1}$ glucose had resulted in excess glucose in the culture. Therefore, this concentration of glucose was used. All remaining components of NMM-J were unaltered.

Vessels of total volume of 11litres were used as reservoirs for 10litres of phosphate-limited NMM-J and nitrogen-limited NMM-J. The media were autoclaved and cooled to room temperature before the addition of sterilised glucose and phosphate. However, addition of phosphate resulted in formation of a yellow-orange precipitate; this had not been observed in previous batch culture experiments with flasks and the Bioengineering fermenter. Precipitation of phosphate can occur at high temperatures, but, in this case, was observed at room temperature possibly because of the large volume (10litres) of the media. Determination of the phosphate concentration (section 2.10.5) in the phosphate- and nitrogen-limited media showed that the concentrations of available phosphate had been reduced from $38mg.l^{-1}$ to $8.7mg.l^{-1}$ and from $190mg.l^{-1}$ to $53.6mg.l^{-1}$ respectively. An attempt was therefore made to reduce this loss in available phosphate. The magnesium sulphate was reduced from $1.0g.l^{-1}$ to $0.8g.l^{-1}$ and, since sulphate was in excess, the concentration of sodium sulphate was reduced from $5.0g.l^{-1}$ to $1.6g.l^{-1}$. Media containing these modifications were sterilized in the absence of the trace elements which were then added after the addition of phosphate. This resulted in a noticeable reduction in the amount of precipitate. Analysis of these media revealed the correct phosphate concentrations.

### 3.7.2 Pre-cultivation

Inoculation of fermenters for batch cultivation, or chemostats for continuous cultivation, is usually performed by the addition of a seed mycelial culture grown in complex or semi-defined media. For these studies, it was not feasible to grow *S. coelicolor* in such media because of the difficulty in stating what kind of metabolism might have occurred. Preliminary attempts to inoculate the Bioengineering fermenter with a mycelial inoculum grown in NMM-J had resulted in severe pelleting. However, an attempt was made to grow a pre-culture for the chemostat in a round-bottomed shake flask. 100ml of phosphate-limited NMM-J (with reduced magnesium and sulphate concentrations) was inoculated with fresh spores at a concentration sufficient to give $1x10^7$ spores per ml of the final chemostat volume. It has been suggested that an initial high $CO_2$ concentration is necessary for successful spore germination and growth (Bader, 1986). This was accomplished by incubating the flask, without shaking, for 5 hours after inoculation. The flask was then incubated on a reciprocal shaker for a further 18 hours by which time the germ-tubes and some hyphae were visible. Inoculation of the chemostat with the young mycelia, some of which were aggregated, resulted in the formation of tight pellets. Two additional pre-cultures gave the same result. Fresh spore inocula were therefore used and germination was allowed to occur under aerated conditions, but in the absence of agitation and flow of medium.

### 3.7.3 Steady State

As outlined in the description of continuous cultivation (section 1.5.2), steady-state is a period in which the growth rate and nutrient concentrations are constant, allowing the maintenance of a metabolic steady-state. The timescale for the attainment of steady-state is given by the number of dilutions of the volume in the chemostat vessel. That is, if a vessel contains a fixed volume of 1litre and the culture requires a total volume of 2litres to have flowed through the vessel in order to reach steady-state, a maximum of two dilutions are required to reach this steady-state. In this experiment, five dilutions occurred prior to steady-state measurements of biomass being made. One dilution resulted in 50% of the original inoculum remaining. A second dilution removed another 50% of that culture, leaving 25% of the original inoculum. A third, fourth and fifth dilution resulted in 12.5%, 6.25% and less than 4% of the original inoculum

remaining. The final value is within the range of analytical errors, therefore any discrepancies in. *e.g.*, the time of germination, are obviated after the fifth serial dilution.

### 3.7.4 Morphological Observations

Samples from the chemostat were checked, using a Zeiss microscope (Jena), for alterations in mycelial morphology. Aliquots from samples showing interesting morphology were fixed with 1% (v/v) formaldehyde. However, this caused the mycelia to aggregate and form uniform clumps which did not reflect the morphological state of the mycelia on removal from the chemostat. The photographs in figure 3.10 were therefore taken using fresh samples. Following inoculation of the chemostat, the mycelia underwent a period of batch-like growth prior to reaching the growth rate limitation set by the phosphate concentration, at a dilution rate of $0.04h^{-1}$. During this "batch" period, the mycelia were present as semi-dispersed and dispersed aggregates similar to those found in genuine batch cultures. As the growth rate became equivalent to the dilution rate, the majority of the mycelia were single with long, thin apical hyphae, revealing no greater than two short branches. This observation possibly signified extreme nutrient limitation and low growth rates (M. Roth, personal communication; section 1.5.3). An increase in the dilution rate to $0.06h^{-1}$ and then to $0.09h^{-1}$ in this cultivation led to an increase in the number and lengths of the branches, thus increasing the number of apical tips. Loosely entangled aggregates with long hyphae were observed, although well-separated mycelia with a few short branches were also present (figure 3.10a). At a dilution rate of $0.12h^{-1}$ in the same cultivation, the branches had grown longer and were more numerous thus allowing a greater extent of aggregation (figure 3.10b). The mycelial forms could be described as very loose pellets but they did not resemble the relatively dense pellets obtained during batch growth of *S. coelicolor* in NMM-J plus glass beads (section 3.4). Small aggregates were present at this dilution rate too but their hyphae were also very long and thin, possibly as a consequence of nutrient limitations.

To check for mutational changes often observed in continuous cultivations, colony morphologies were examined regularly by inoculating plates of soya-mannitol agar with appropriately diluted samples from the chemostat. At all times, the majority of the colonies continued to produce aerial hyphae

and spores. However, brown mutants began to appear from 550 hours after inoculation. The brown colonies exhibited apparent *bld* phenotypes, but were less than 5% of the colony-forming populations (CFUs) examined.

### 3.7.5 Production of secondary metabolites in the chemostat

To determine how growth in a phosphate-limited culture affected antibiotic production, patterns of actinorhodin production were examined at increasing dilution rates (figure 3.11). Actinorhodin was detectable in the medium at all dilution rates tested. At dilution rates of $0.04h^{-1}$, $0.06h^{-1}$ and $0.09h^{-1}$, production was observed prior to the attainment of steady-state. Concentrations of actinorhodin (section 2.11) reached a maximum followed by a reduction in production as the cells reached a metabolically-balanced state. However, the maximal concentrations were not similar at each dilution rate. The initial peak in concentration of actinorhodin, at a dilution rate of $0.04h^{-1}$, was $36.2\mu g.ml^{-1}$, while the maximum concentrations determined at $0.06h^{-1}$ and $0.09h^{-1}$ were $10.3\mu g.ml^{-1}$ and $8.2\mu g.ml^{-1}$ respectively. Yields of actinorhodin (in $\mu g.g^{-1}$ of dry weight biomass) were not determined because biomass concentration was not measured (see section 3.7.6). However, samples taken from the chemostat at various times after production of actinorhodin had begun were diluted and grown on soya-mannitol agar. The number of colony forming units (CFUs) were counted (as for spore counts, section 2.4.4) after 3 to 4 days. The concentration of CFUs at dilution rates of $0.04h^{-1}$, $0.06h^{-1}$ and $0.09h^{-1}$ were $1.2x10^{6}cfu's.ml^{-1}$, $2.9x10^{5}cfu's.ml^{-1}$ and $1.5x10^{6}cfu's.ml^{-1}$ respectively. However, it was not possible to estimate the biomass concentrations from these figures, because mycelia had grown in a filamentous fashion at the dilution rate of $0.04h^{-1}$, whereas they had been observed to aggregate at $0.09h^{-1}$.

At a dilution rate of $0.09h^{-1}$, a decrease in biomass density occurred as observed by the volume of mycelia settled at the bottom of the sample tube. The extent of pelleting also increased at this time. These phenomena occurred simultaneously with an increase in the mycelial growth on the vessel wall. Exchange of reservoirs resulted in a lag phase of 20 to 30 hours which was followed by a decrease in the residual glucose concentration (figure 3.11). Utilization of glucose at this time perhaps reflected an increase in production of biomass. Actinorhodin was produced as the concentration of glucose tended towards a constant level. The increase in the peak height

of actinorhodin, as compared to that observed previously at $0.09h^{-1}$, may have been due to the presumed increased biomass concentration ($5.18x10^6$ $CFUs.ml^{-1}$ were determined) or to actinorhodin produced by mycelia which had been growing on the siliconized vessel wall. Occasionally, small clumps of mycelia fell from the wall into the culture. Actinorhodin was also observed in the medium while pH and glucose measurements were constant. Constancy of these parameters had been used previously to indicate steady-state. Again production of actinorhodin may have been attributable to the mycelia coating the inside wall of the chemostat vessel.

In addition to the production of actinorhodin, mycelially-associated orange and yellow pigments were observed throughout the fermentation. The orange pigment was possibly the result of the simultaneous production of undecylprodigiosin, which is red, and the yellow pigment. Undecylprodigiosin was produced at times coincidental with the secretion of actinorhodin into the medium. The yellow pigment, however, was observed to be present at times of production of both of the coloured antibiotics and also during the periods of steady-state at each dilution rate used. This may have indicated the production of undecylnorprodigiosin, the penultimate product of the *red* biosynthetic pathway, which is also yellow (Feitelson *et al.*, 1985). The pigment was not proven to be undecylnorprodigiosin, however, and so for the purpose of further discussion is termed" yellow".

Although quantitative determinations of undecylprodigiosin were not made, it was observed that on increasing the dilution rate from $0.04h^{-1}$ to $0.06h^{-1}$ and $0.09h^{-1}$ the intensity of the yellow decreased at each steady-state. At $0.12h^{-1}$, there was continuous production of undecylprodigiosin without yellow, as indicated by a light red colouration. Production was concomitant with actinorhodin production.

In an attempt to determine if actinorhodin was produced in nitrogen-limited conditions, a preliminary continuous culture was set up . NMM-J was modified, as described in section 3.7.1, to give a nitrogen-limited medium. However, continuous cultivation of *S. coelicolor* 1147 in this medium did not result in actinorhodin production. Colonies of cells derived from this culture were able to produce actinorhodin when grown on plates of soya-mannitol agar, demonstrating that the majority of the cells maintained the ability to produce the antibiotic under appropriate conditions.

Undecylprodigiosin and yellow were produced at similar stages of growth in the nitrogen-limited medium as in the phosphate-limited culture, *i.e.* both red and yellow pigments were produced simultaneously prior to steady-states at dilution rates of $0.02h^{-1}$, $0.05h^{-1}$ and $0.09h^{-1}$. The production of yellow was also observed to continue throughout steady-states. However, both pigments seemed to be produced at intensities higher than those observed in the phosphate-limited culture.

### 3.7.6 Phosphate utilization

Determination of the concentration of phosphate (section 1.10.5) in a preliminary continuous cultivation showed that, during steady-state at dilution rates of $0.02h^{-1}$ and $0.05h^{-1}$, available phosphate was at a level of less than $0.10mg.l^{-1}$ to $0.21mg.l^{-1}$ (less than 1μM to 2μM). The fermentation, from which the data for figure 3.11 were obtained, was carried out based on these phosphate results and chemostat samples were stored and shipped in dry ice to Glasgow for further analysis. However, due to unforeseen circumstances the samples remained at Berlin Lichtenberg Airport for 6 days. On arrival, biomass samples were washed with 1ml of 10mM $KH_2PO_4$, pH7.0, and the supernatants were checked for lysis, as measured by carbon analysis. The supernatants contained high concentrations of carbon indicative of lysis:

| biomass (ppm) | supernatant (ppm) |
|---|---|
| 1411 | 558.4 |
| 1897 | 1367.5 |
| 4497 | 2848.9 |

This rendered the results from any further analysis doubtful since it was not possible to state what alterations may have occurred within the samples. However, figure 3.11 was composed of results from glucose analyses, pH measurements and actinorhodin determinations carried out during the cultivation in Jena.

Figure 3.12 shows the pattern of phosphate utilization in the phosphate-limited continuous culture described in section 3.6.5, determined (section 2.10.5) after lysis, in Glasgow. Quantitative scrutiny is not possible but the pattern of phosphate concentration followed that of glucose utilization (as

measured by the method in section 2.7.4.1), and an increase in the available phosphate level coincided with a reduction in actinorhodin production.

## 3.8 Discussion

Various methods were used in the attempt to obtain dispersed mycelia which produced actinorhodin at reproducible times and of similar concentrations. Chemical agents had fulfilled these requirements by either increasing the viscosity of the medium or causing electrostatic repulsion between hyphae. However, these methods interfered with the determination of carbon and, consequently, were not useful.

Preliminary comparative studies of *S. coelicolor* 209 growth with different mechanical means of dispersal favoured the use of glass beads. In their experiment, Doull and Vining (1989) had compared the growth of *S. coelicolor* in a medium containing glass beads in the presence and absence of starch. The method involving the use of glass beads alone was the least productive in terms of both yield of biomass and production of actinorhodin. They had used approximately 100, 0.3cm diameter glass beads per 250ml flask. The shearing capacity of the beads may have severed hyphae in peripheral growth zones, leading to the loss of cytoplasm and cell death (Bader, 1986) which may provide an explanation for the low yield of biomass. Growth and biomass yield may have also been affected by the presence of the glass beads at the time of inoculation. Section 3.3.1 demonstrates the potential requirement for the germination of *S. coelicolor* 209 spores prior to the addition of glass beads. The presence of starch in the medium of Doull and Vining (1989) may have protected the hyphae from the shearing action of the beads.

Sufficient levels of *S. coelicolor* biomass were important for further compositional analyses in this study. These were not obtainable from individual 50ml cultures of *S. coelicolor* 209. A reason for this may have been the use of nitrate in the medium as the nitrogen source. The reduction of nitrate into utilizable ammonia requires a high energy consumption: one molecule of nitrate needs four pairs of reducing equivalents for reduction. The $NH_4^+$ thus formed is converted to glutamate by means of the glutamine synthetase and glutamate synthase coupled reactions (Brown *et al.*, 1974; section 1.9.3), which require the hydrolysis of one ATP molecule. In contrast, $NH_4^+$ from a source in the medium is assimilated by the action of

glutamate dehydrogenase which needs only a reducing agent and has a low affinity for $NH_4^+$ (section 1.9.3). Thus the enzyme is not saturated under limiting conditions of $NH_4^+$. Therefore, use of nitrate as the nitrogen source undoubtedly has consequences for the amount of carbon source and energy, as ATP, available for the production of cellular biomass.

Quantities of biomass were increased by the pooling of cultures grown to the same degree of antibiotic production. Variations in the macromolecular compositions of biomass from individual and pooled cultures, which were not averaged-out by pooling, indicated that a similar extent of antibiotic production did not necessarily signify similar macromolecular compositions. However, analysis of portions of the same biomass sample did show that the analysis techniques for the macromolecular composition were reproducible (section 4.4.1). Cultures of *S. coelicolor* 209, harvested at the same time after inoculation, showed closer similarities in composition. Pooling of cultures was also disadvantageous because it led to heterogeneous populations of cells.

In cultures of *S. coelicolor* 209, production of actinorhodin varied between mycelia grown from different spore stocks and within the same spore stock. This may have been due to a number of factors, *e.g.*, freezing of the vulnerable pre-germinated spores, or to minute, undetectable alterations in the medium or in agitation or aeration. Discrepancies in the production of actinorhodin were also observed using fresh spores of *S. coelicolor* 1147, although, in general, production in flasks was reproducible 48 to 60 hours after inoculation.

Batch cultivations of *S. coelicolor* 1147 in the Bioengineering fermenter gave sufficient quantities of biomass for the analyses described in chapter 4. However, biomass yields were not good since 4g of glucose were utilized to produce a maximum amount of 1g dry weight of biomass: a yield of approximately 25%. Fermentations of *S. thermonitrificans* in the same fermenter had produced yields of 78% (Burke, 1991). Variations, similar to those in flask cultures, also occurred in fermenter cultures which mainly concerned biomass production. Culture conditions were the same during each fermentation (except when extra glucose was added), but growth of *S. coelicolor* in NMM-J was, in general, not reproducible between five fermentations.

Under the conditions used by Hobbs *et al.* (1989) and Doull and Vining (1989), actinorhodin was produced in the stationary phase in batch cultures, with the carbon source as limiting factor. In these studies, an excess of both glucose and phosphate was present in both flask and fermenter cultivations of *S. coelicolor* 209 and 1147 at the time of production of actinorhodin (nitrate was also shown to be present in excess during phosphate-limited continuous cultures). This suggests that nutrients other than these are the limiting factors and are involved in the switch from primary to secondary metabolism. However, the cells may detect a reduction in concentration of any of these three major nutrients which is indicative of a subsequent nutrient-limitation. Metabolic readjustment in the form of secondary metabolism may occur as a preparation for this time which is also a period of morphological differentiation on solid media and, therefore, vulnerability. In the fermenter cultivations, actinorhodin was also produced while the biomass was still increasing. Although wet weight measurements were later found to be unsuitable for the determination of biomass, a further increase in dry weight measurements after production of actinorhodin suggested that actinorhodin was produced during the transient phase between growth and stationary phases. Transcripts from the *actIII* gene have been detected during the period prior to stationary phase (Strauch *et al.*, 1991), indicating that transcription of this gene and perhaps the whole *act* cluster is active at this time.

An apparent alteration in morphology was observed in fermenter cultivations at the time of production of actinorhodin: pellets became more dispersed and floccular. This may have been the result of lysis, although small mycelial fragments which are indicative of lysis were not observed. Morphological changes are known to coincide with metabolic changes of *Streptomyces* on solid media, *e.g.*, aerial hyphae formation coincides with secondary metabolite production. Sporulation and aerial hyphae formation are rarely observed in liquid cultures of *S. coelicolor*, although they do occur in some *Streptomyces* species (section 1.5.3). However, it is not possible to compare morphological alterations in submerged culture with those on solid media.

The continuous culture of *S. coelicolor* 1147, at low growth rates in a phosphate-limited medium, resulted in dispersed growth to an extent unobtainable in batch cultures. Mycelia grew individually with very few branches at an initial dilution rate of $0.04h^{-1}$. The presence of nitrate as the nitrogen source may have contributed to the pattern of growth in the

chemostat, a pattern which indicated very strong limitations. Individual mycelia were observed at a dilution rate of $0.09h^{-1}$, although mycelial entanglement also occurred.

Continuous cultivation of some unicellular organisms over long periods of time acts as a selective pressure for mutants with enhanced rates of nutrient uptake which exhibit faster rates of growth. However, continuous cultivation of filamentous organisms could also act as a selective pressure for a fragmented mycelial morphology (section 3.1.1). Mutants with highly dispersed phenotypes were obtained by the prolonged cultivation of *S. lividans* (D. Noack, personal communication). These mutants sedimented slowly in comparison to the wild type strain, and had an altered composition of membrane lipid. Colonies exhibited a change in morphology on solid media and had lost the ability to form aerial hyphae and spores. Undecylprodigiosin was produced in bright red colonies, but there was no observable production of actinorhodin. Mycelial variants were also observed in the continuous cultivation of *S. thermonitrificans* (Burke, 1991).

Colonies grown from samples removed from the continuous culture of *S. coelicolor* 1147 did not show any alterations in morphology until approximately 550 hours after the inoculation of the chemostat. From that time, until the end of the cultivation, a few mutants exhibiting *bld* phenotypes were observed. At no time, however, did they form the majority of the population.

An interesting feature of the continuous cultivation was the period of actinorhodin production. Actinorhodin was produced at times just before the cells reached their metabolic steady-state at all the dilution rates tested. The transient phase between steady-states is very complex metabolically: cells grow in almost batch-like conditions until the growth rate is limited by the low concentration of the limiting nutrient. Steady-state is the most useful period in continuous cultures, but dilution rates low enough for the continual production of actinorhodin, if any, were not tested.

During the transient period between growth and stationary phases, a metabolic imbalance may occur, during which cellular components are synthesized at different rates. Such an imbalance can result in the formation of metabolites unnecessary for growth. It is hypothesized that actinorhodin was produced during such a transient period, *i.e.*, as the phosphate

limitation was reached with the carbon source in excess. To balance growth, *i.e.*, reach steady-state, a metabolic readjustment may have taken place in the form of production of actinorhodin.

The differences in peak heights of production of actinorhodin at each dilution rate may have been attributable to the concentration of phosphate available in the medium. Phosphate concentration was not measured at the time (see section 3.7.6 for explanation). It is therefore not possible to describe the changes in phosphate concentration. Theoretically, however, in a phosphate-limited culture which exhibits no measurable phosphate during steady-state, an increase in dilution rate would increase the available concentration of phosphate until the subsequent steady-state was attained. The concentration of phosphate would be negligible at this time. This would cause a change in the metabolism and growth rate of the cells, resulting in a metabolic imbalance as the cells progressively reached steady-state. The new dilution rate and the previous one may have affected the degree of imbalance in the system.

At a dilution rate of $0.04h^{-1}$, the cells had undergone a previous state of batch-like growth in a phosphate sufficient medium to obtain sufficient quantities of biomass. On switching to the phosphate-limited medium, at $0.04h^{-1}$, the low flow rate may have resulted in the availability of the phosphate for a longer time than if the dilution rate had been increased from $0.02h^{-1}$ to $0.04h^{-1}$. The batch-like growth and higher phosphate concentration may have contributed to a more severe metabolic imbalance, resulting in the production of a higher concentration of actinorhodin. Increasing the dilution rate from $0.04h^{-1}$ to $0.06h^{-1}$ may not have resulted in an imbalance to such an extent. Consequently, there was less production of actinorhodin.

The increase in peak height at dilution rates of $0.09h^{-1}$ and $0.12h^{-1}$ may have been caused by the measurement of actinorhodin produced by small clumps of mycelia which had fallen from the walls of the vessel, where it had been growing. Alternatively, an increase in biomass may have resulted in increased production of the antibiotic.

Undecylprodigiosin and a yellow pigment, possibly undecylnorprodigiosin, were also produced during the phosphate-limited cultivation. Production of undecylprodigiosin is growth related, being produced late in growth phase in batch cultures; it is not observable in stationary phase. The exact fate of

the red pigment, however, is not known. For example, it may be swamped by actinorhodin, modified to a colourless product, or degraded and utilised as a precursor for actinorhodin biosynthesis. The yellow pigment was not observed at any time in batch cultures. Hobbs *et al.* (1990) mentioned that undecylprodigiosin was produced during steady-state in continuous culture under conditions of glucose limitation, at concentrations inversely proportional to growth rate.

However, production of undecylprodigiosin occurred simultaneously with production of actinorhodin under phosphate-limited conditions, perhaps as an additional indicator of metabolic imbalances. Yellow was produced throughout steady-state and also during the same period of production as the antibiotics. Undecylnorprodigiosin differs from undecylprodigiosin by a methyl group, and the conversion to undecylprodigiosin is by undecylnorprodigiosin: S-adenosylmethionine *O*-methyltransferase. If yellow was undecylnorprodigiosin in these studies then, interestingly, the rate-limiting step in the *red* biosynthetic pathway, during steady-state conditions, would be the transferase. This suggests that phosphate may be involved in the metabolic control of undecylprodigiosin biosynthesis.

Nitrogen-limited cultures also produced undecylprodigiosin and yellow. However, there was no determinable production of actinorhodin. The red and yellow pigments were observable in the same time patterns as in the phosphate-limited culture. Nitrogen may also be involved in the control of the *red* pathway but the question which arises is: why would an antibiotic biosynthetic pathway which utilizes nitrogen be switched on at times of nitrogen limitation? Nitrogen may be available for production of undecylprodigiosin during periods of metabolic imbalance, but, unless yellow is not undecylnorprodigiosin, the reasons why are not clear.
Lack of production of actinorhodin during nitrogen limitation suggests that the concentration of phosphate was too high at the low growth rates to permit production, whereas the nitrogen concentration in the phosphate-limited culture did not affect production. Undecylprodigiosin was produced under both phosphate and nitrogen limiting conditions, indicating the use of different control mechanisms in the biosynthesis of the red antibiotic and actinorhodin.

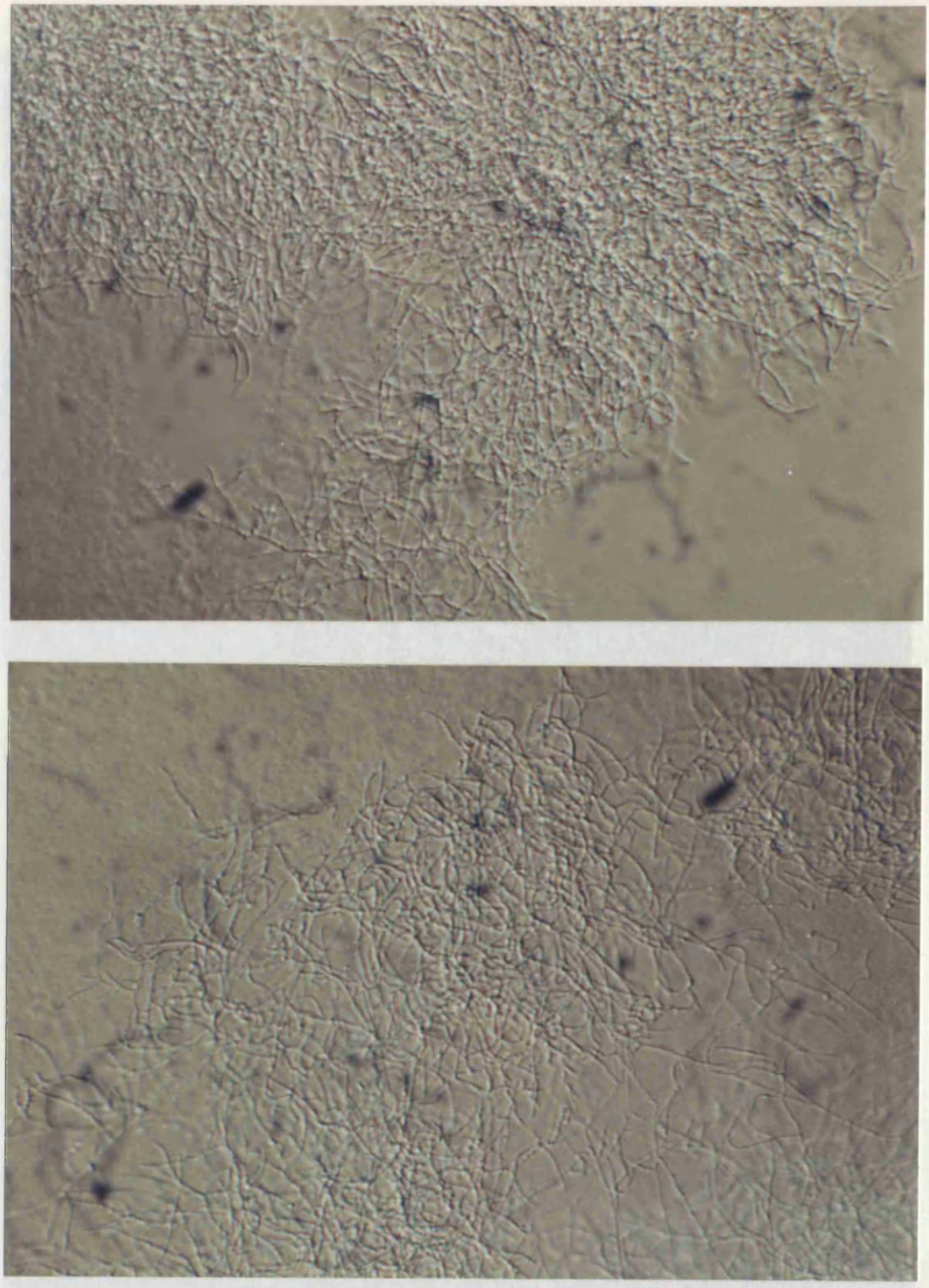


**Figure 3.1. Morphological forms of *S. coelicolor* 209 grown under differing conditions in flask cultivations.**

Pre-germinated spores of *S. coelicolor* 209 were inoculated into 50ml of HMM (Hobbs *et al.*, 1989) or NMM-J (section 2.5.1) in 250 ml flasks to give a final concentration of spores of $5x10^5$ spores.ml$^{-1}$. The flasks were incubated in an orbital shaker at 30°C for 72 hours. Samples were removed and prepared on glass slides. Photographs were taken using Nomarski optics (magnification X312.5). **a.** Morphology of *S. coelicolor* 209 grown in HMM. The mycelia were extremely dispersed and individual hyphae may be seen. A "halo" of junlon could also be seen among the hyphae.

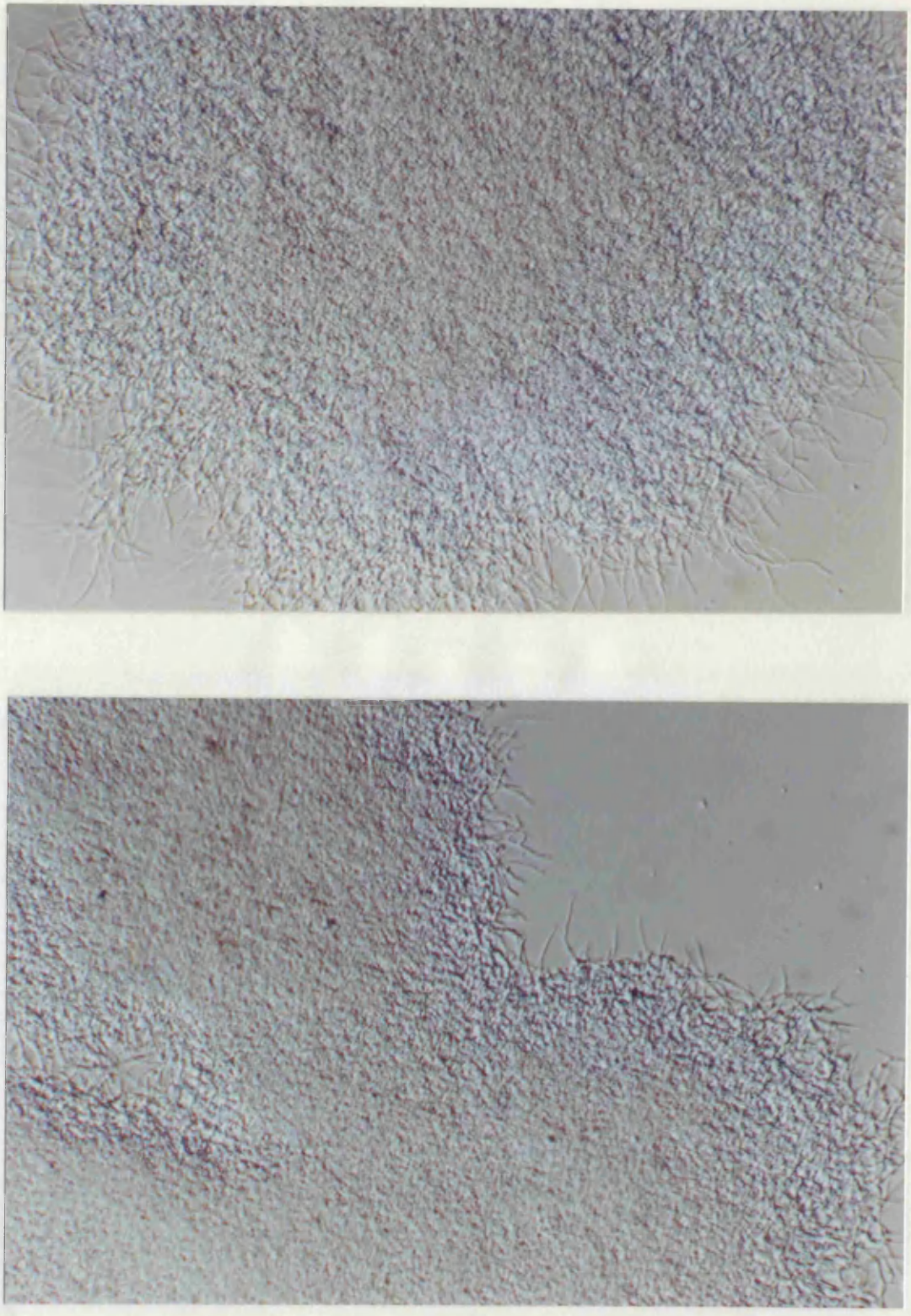


**Figure 3.1** continued. **b**. Morphology of *S. coelicolor* 209 grown in NMM-J. The dense central areas exhibited red pigmentation indicative of the production of undecylprodigiosin. The peripheral regions contained younger mycelia which had not produced the antibiotic.

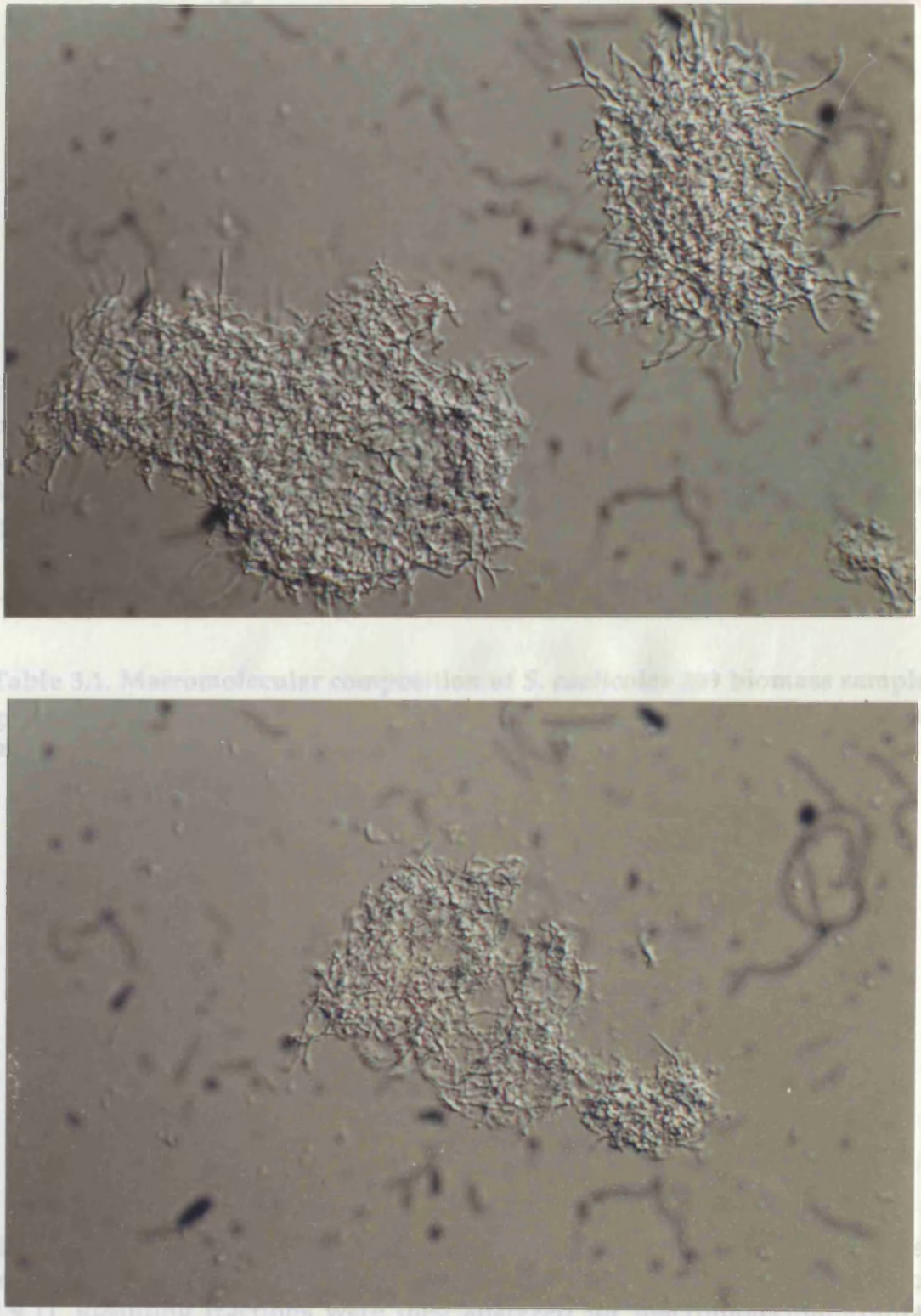


**Figure 3.1** continued. **c.** Morphology of *S. coelicolor* 209 grown in NMM-J plus glass beads. The pellets were much smaller than those observed in NMM-J without the shearing action of the glass beads. However, they still contained relatively dense central regions. The pellets also had a "battered" appearance with some hyphae visible in the centre.

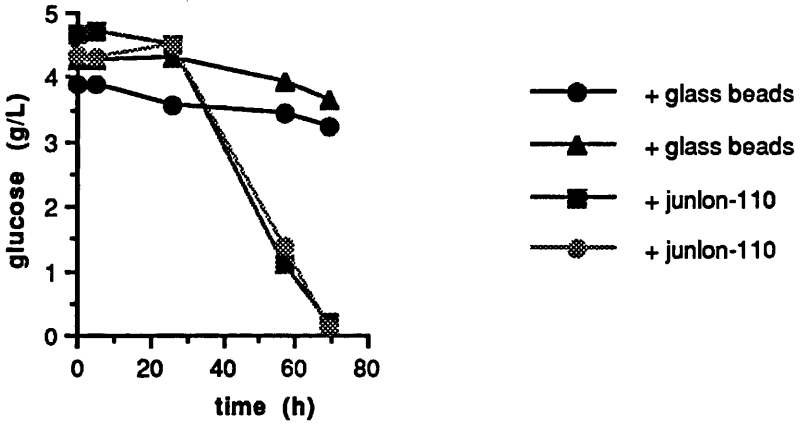



**Figure 3.2. Glucose utilization by *S. coelicolor* 209 in the presence of glass beads or junlon-110.** Duplicate cultures from spore concentrations of $5x10^5$ spores.ml$^{-1}$ were grown in 50ml NMM-J plus glass beads or in 50ml HMM. Supernatant samples were removed at indicated times and analyzed for glucose concentration (section 2.7.4.1).

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**Table 3.1. Macromolecular composition of *S. coelicolor* 209 biomass samples grown in shake flasks.** All macromolecular values are in mg.g$^{-1}$ dry weight biomass.

| SAMPLE | TIME (h) | CHO | RNA | DNA | PROTEIN |
|---|---|---|---|---|---|
| $A^r$ | 73.7 | 9.1 | 83.6 | 36.6 | 166.0 |
| $B^r$ | 73.6 | 8.2 | 74.7 | 42.6 | 168.4 |
| $C^r$ | 39; 66 | 10.6 | 112.5 | 22.5 | 172.5 |
| $D^r$ | 30.5; 60.5 | 11.7 | 142.2 | 32.0 | 288.3 |
| $E^a$ | 95.5 | 14.0 | 120.6 | 11.0 | 369.3 |
| $F^a$ | 116.25; 121 | 12.1 | 129.4 | 25.4 | 202.9 |
| $G^w$ | 101 | 9.5 | 123.3 | 34.8 | 232.0 |
| $H^w$ | 93 | 5.2 | 132.6 | 12.5 | 20.0 |

Biomass grown in 50ml NMM-J was harvested at the stated times and subjected to a fractionation method for the separation of cellular macromolecules (section 2.9.1). Resulting fractions were then analyzed for macromolecular content (section 2.10) and each macromolecule was expressed as mg.g$^{-1}$ dry weight of biomass. Two time points corresponding to the same sample show that the biomass in that sample resulted from pooled cultures of similar antibiotic production. CHO, carbohydrate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid. a indicates production of actinorhodin, r indicates production of undecylprodigiosin and w indicates lack of antibiotic production. N.B., protein weights do not account for 50% of the biomass. See section 4.4.3 for an explanation.

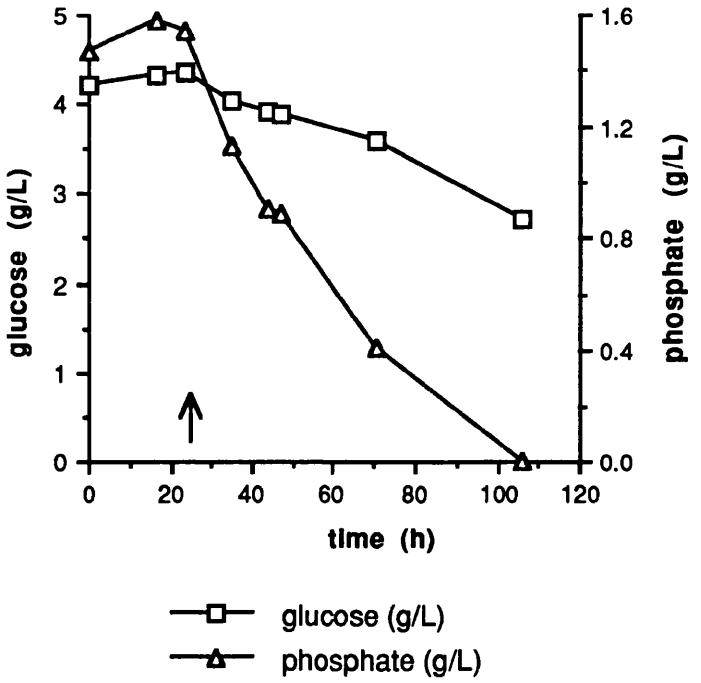


**Figure 3.3. Glucose and phosphate utilization by *S. coelicolor* 1147.**
Mycelia, grown from a fresh spore suspension of concentration $1x10^7$ spores.ml$^{-1}$ of culture, were cultivated in 50ml of NMM-J plus glass beads. Samples taken throughout the cultivation were assayed for glucose (section 2.7.4.1) and phosphate (section 2.10.5). The arrow denotes time of actinorhodin production. Values are the means of triplicate cultures.

**Figure 3.4. Morphological forms of *S. coelicolor* 1147 grown under differing conditions in flask cultivations.**
Duplicate cultures of *S. coelicolor* 1147 were grown in three different media conditions from concentrations of $1x10^7$ spores.ml$^{-1}$: HMM (Hobbs *et al.*, 1989), NMM-J (section 2.5.1) and NMM-J plus glass beads. The cultures were incubated in an orbital shaker at 30°C for 72 hours. Samples were removed and prepared on glass slides. Photographs were taken using Nomarski optics (magnification X312.5). **a.** Morphology of *S. coelicolor* 1147 grown in HMM. The mycelia were well separated thus allowing optimal diffusion of nutrients.

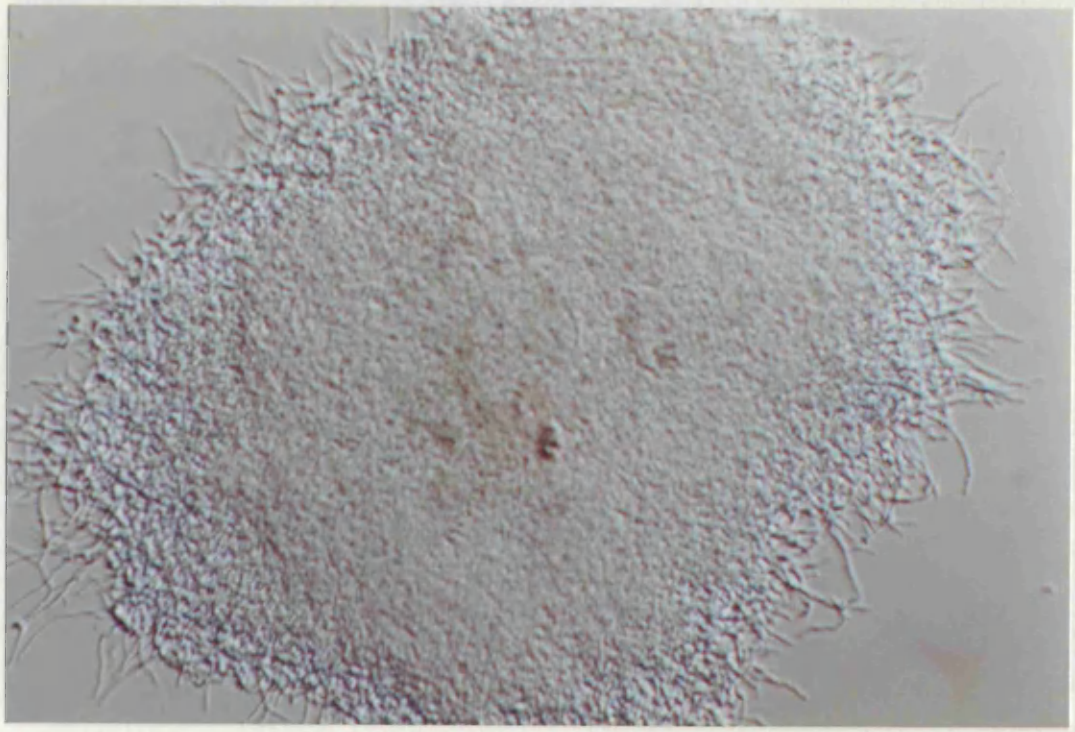

**Figure 3.4** continued. **b**. Morphology of *S. coelicolor* 1147 grown in NMM-J. Very large, dense pellets were observed which were subject to nutrient limitations. The heterogeneity of the pellets was indicated by the production of undecylprodigiosin in the centres.

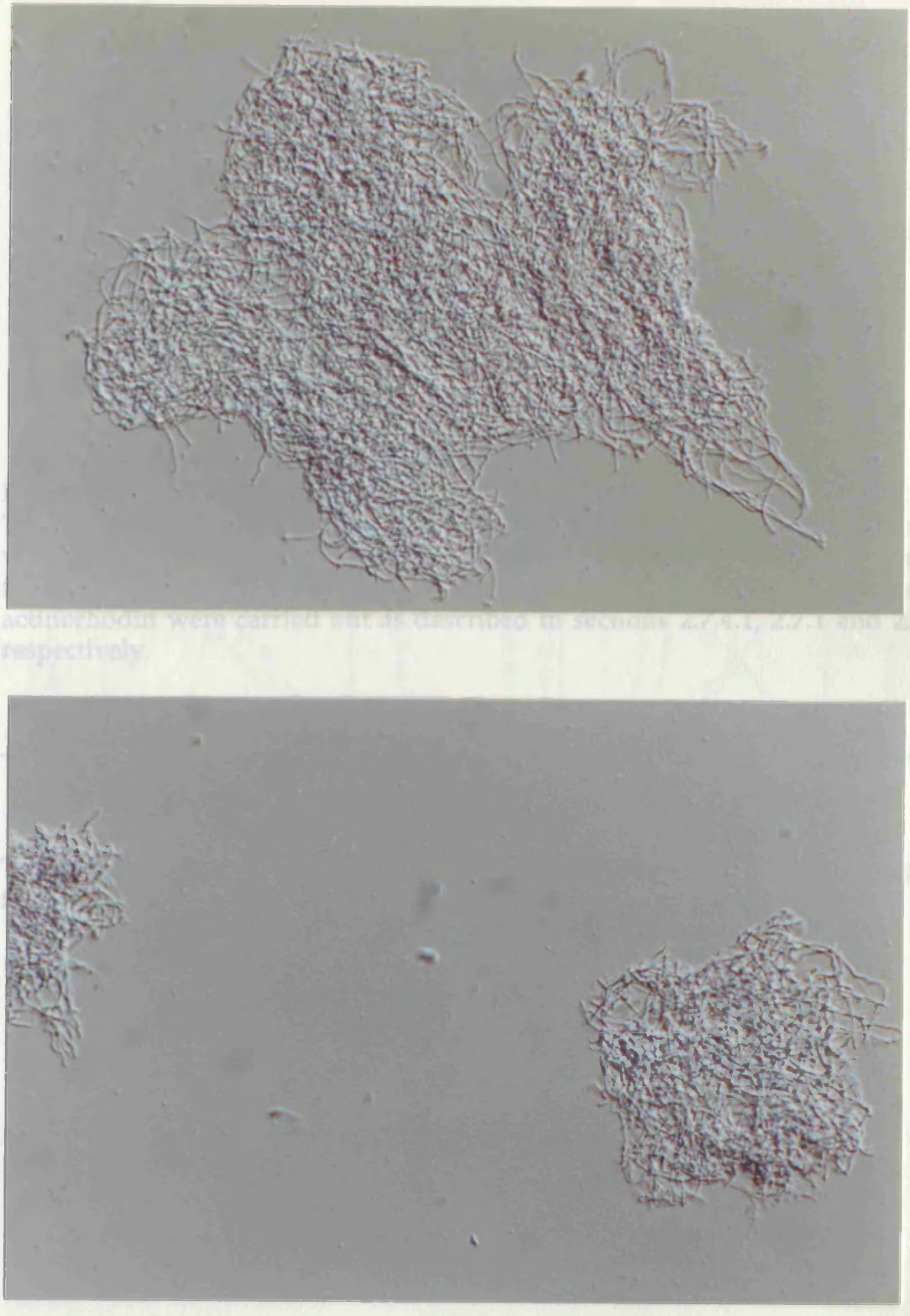

**Figure 3.4** continued. **c.** Pellets were still formed, although they were smaller than those observed in NMM-J without the shearing action of the glass beads, and exhibited a slightly"battered" look. Nutrient limitations would also have occured in these pellets but to a lesser extent since some individual hyphae were visible in the centres.

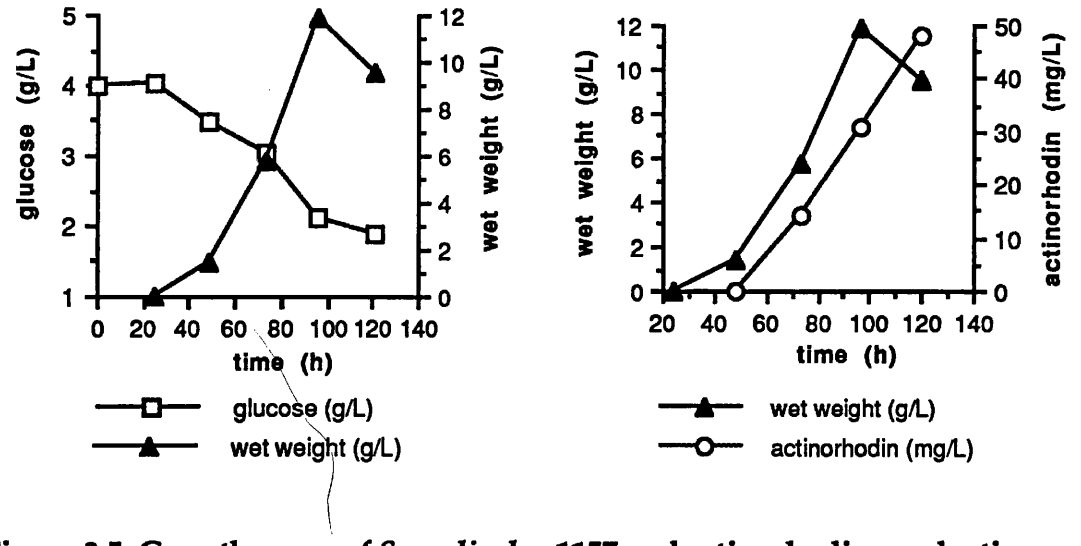


**Figure 3.5. Growth curve of *S. coelicolor* 1157 and actinorhodin production.** Ten cultures of *S. coelicolor* 1147 were grown in 50ml of NMM-J. Three cultures were harvested and pooled for each of the first two points on the graphs, two for each of the last two points. Determinations of glucose, wet weight and actinorhodin were carried out as described in sections 2.7.4.1, 2.7.1 and 2.11 respectively.

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**Table 3.2. Increase in concentration of Tris with time in the Bioengineering fermenter.**

| TIME (h) | TOC (ppm) | TRIS ($g.l^{-1}$)$^x$ |
|---|---|---|
| 0 | 478.6 | 1.21 |
| 1.5 | 457.2 | 1.15 |
| 7.3 | 478.6 | 1.21 |
| 20 | 476.2 | 1.20 |
| 26.25 | 475.2 | 1.20 |
| 30.3 | 524.0 | 1.32 |
| 43.75 | 537.7 | 1.36 |
| 92.5 | 551.7 | 1.39 |

The Bioengineering fermenter containing 7litres of NMM-J minus glucose (the only carbon-containing compound in the medium was Tris) was run for 4 days. Samples were removed and analyzed for organic carbon using the TOCA (section 2.7.3). Results obtained as ppm of carbon were expressed as $mg.ml^{-1}$ of Tris. x, the concentration of Tris (MW 121.1) in NMM-J was $1.2g.l^{-1}$ (10mM) which was equivalent to 480ppm carbon; TOC, total organic carbon as estimated by the TOCA.

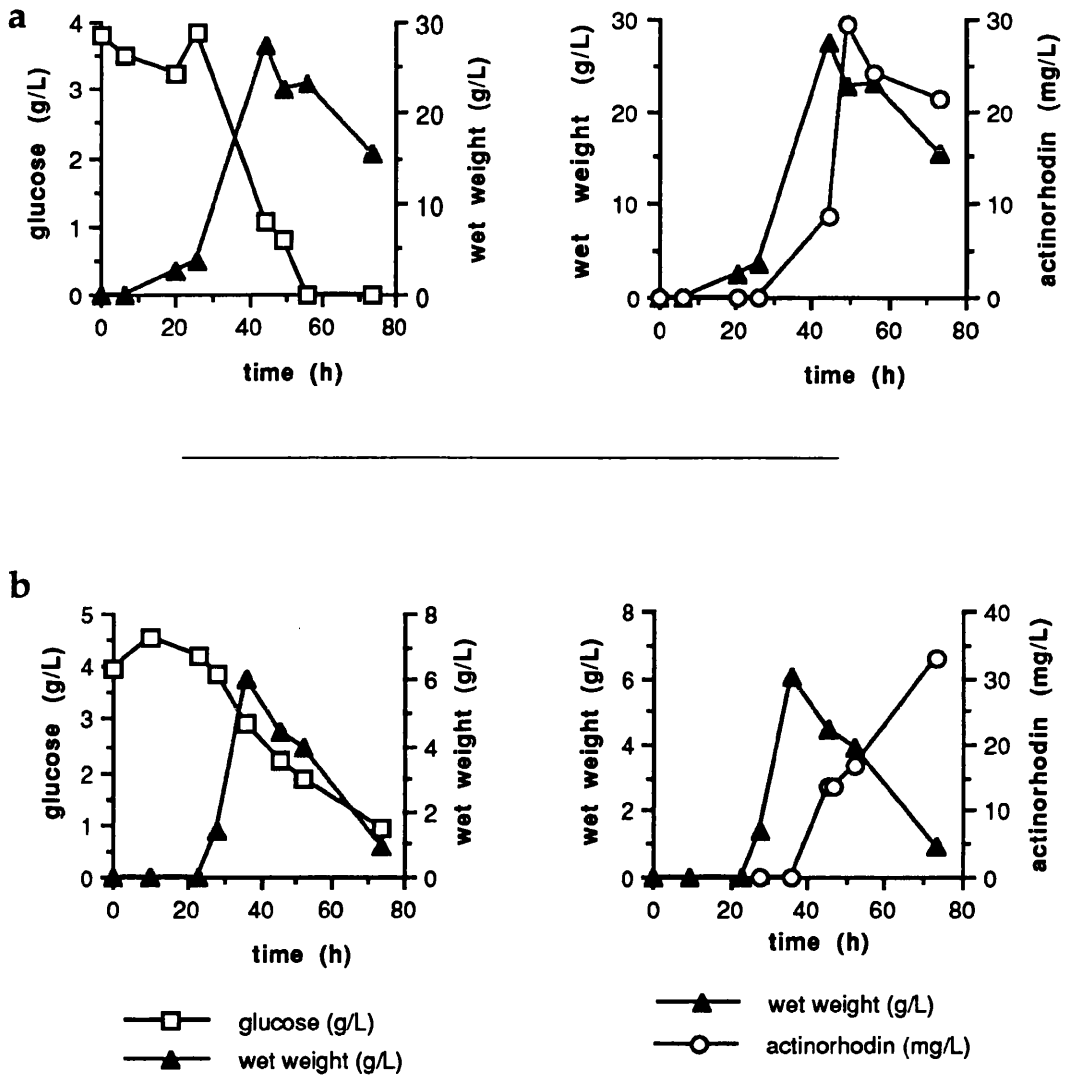


**Figure 3.6. Batch cultivation of *S. coelicolor* 1147 in the Bioengineering fermenter: glucose utilization and production of biomass and actinorhodin.** Five fermentations (**a** to **e**) were carried out using *S. coelicolor* 1147 spores which were inoculated into 7litres of NMM-J in the fermenter at a concentration of $1x10^7$spores.ml$^{-1}$ of culture. Resulting biomass was harvested at specific times during each fermentation: 1litre samples removed up to 50 hours after inoculation, 500ml samples thereafter. Each culture sample was assayed for glucose (section 2.7.4.1) and actinorhodin (section 2.11), and washed biomass pellets were weighed to give wet weight determinations (section 2.7.1). Dry weight in fermentation **e** was determined by the filtration method (section 2.7.2.1). Glucose utilization over time is presented on the same graph with weight of biomass; the change in concentration of actinorhodin is also plotted with weight of biomass.

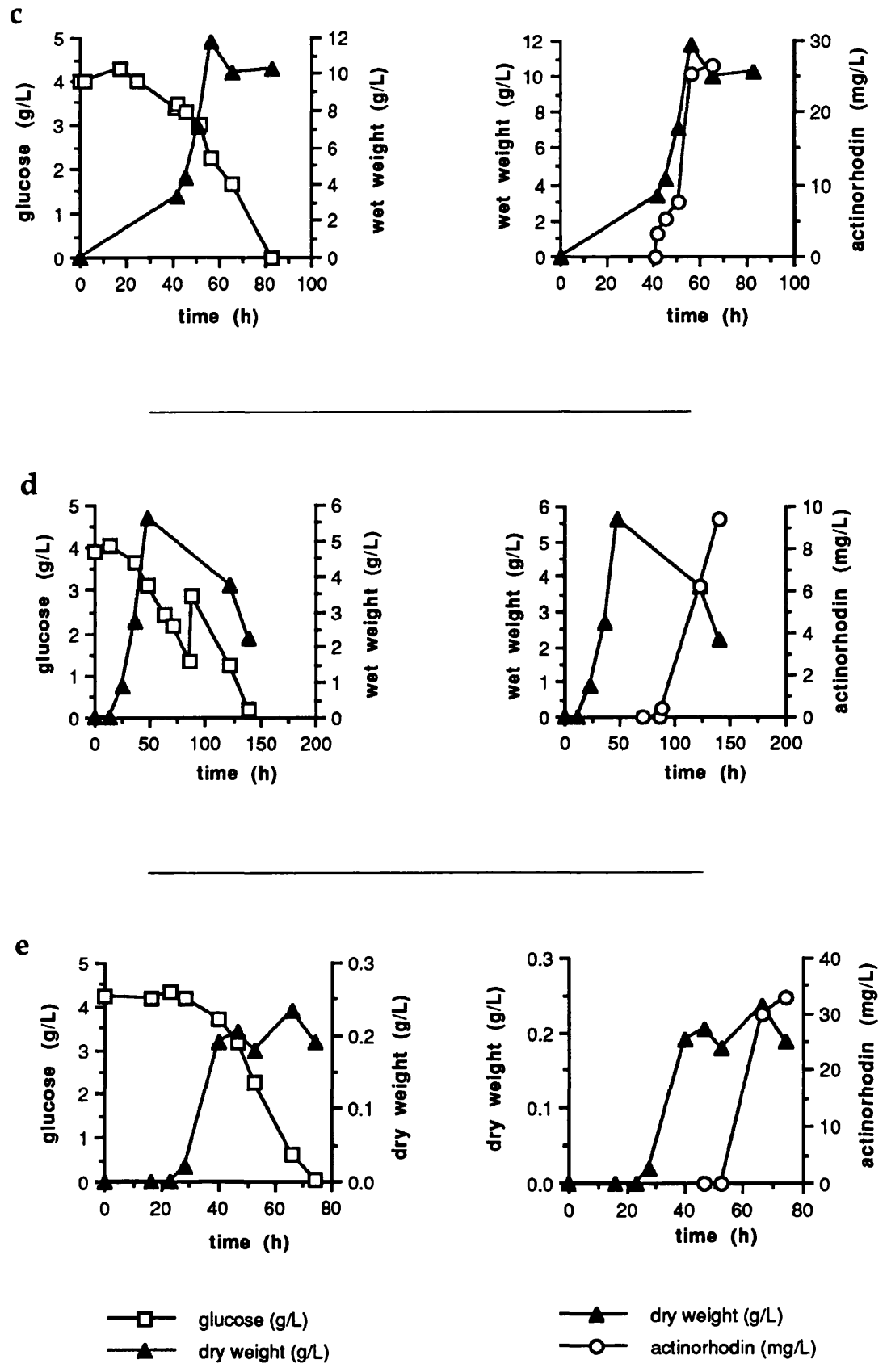


**Figure 3.6** continued.

**Table 3.3. Comparison of wet weight and dry weight estimations of *S. coelicolor* 1147 biomass grown in the Bioengineering fermenter.** All values are in $g.l^{-1}$ culture.

**Fermentation a**

| TIME (h) | WET WEIGHT | DRY WEIGHT[1] | % | DRY WEIGHT[2] | % |
|---|---|---|---|---|---|
| 28 | 1.44 | nd | | 0.04 | 2.8 |
| 35.7 | 6.02 | 0.23 +/- 0.12 | 3.8 | 0.24 | 4.0 |
| 45.8 | 4.44 | 0.25 +/- 0.04 | 5.6 | 0.19 | 4.3 |
| 52.25 | 3.94 | 0.27 | 6.8 | 0.19 | 4.8 |

**Fermentation b**

| TIME (h) | WET WEIGHT | DRY WEIGHT[1] | % | DRY WEIGHT[2] | % |
|---|---|---|---|---|---|
| 41.5 | 3.34 | 0.16 +/- 0.04 | 4.8 | 0.09 | 2.7 |
| 45.2 | 4.29 | 0.08 +/- 0.03 | 1.9 | 0.15 | 3.5 |
| 50.5 | 7.09 | nd | | 0.23 | 3.2 |
| 56 | 11.74 | 0.24 | 2.0 | 0.31 | 2.6 |
| 65.25 | 10.0 | 0.35 +/- 0.02 | 3.5 | 0.37 | 3.7 |
| 82.5 | 10.24 | 0.64 +/- 0.1 | 6.2 | 0.71 | 6.9 |

**Fermentation c**

| TIME (h) | WET WEIGHT | DRY WEIGHT[1] | % | DRY WEIGHT[2] | % |
|---|---|---|---|---|---|
| 20.25 | 2.47 | 0.09 +/- 0.01 | 3.8 | 0.09 | 3.8 |
| 25.75 | 3.62 | 0.13 +/- 0.04 | 3.6 | 0.14 | 3.9 |
| 44.25 | 27.42 | 0.68 +/- 0.04 | 2.5 | 0.83 | 3.0 |
| 49.25 | 22.58 | 1.06 +/- 0.07 | 4.7 | 1.08 | 4.8 |
| 55.75 | 23.11 | 0.82 +/- 0.07 | 3.5 | 0.71 | 3.1 |
| 73.25 | 15.49 | 0.99 +/- 0.05 | 6.4 | 0.42 | 2.7 |

Dry weight measurements of samples from fermentations **a** to **c** (figure 3.6) were determined by two different methods (1 and 2) and expressed as percentages of the wet weights.
1, dry weight measurements as determined by the filtration method (section 2.7.2.1). Measurements are the means of triplicate samples plus standard deviations.
2, dry weight measurements determined by carbon analysis of biomass (section 2.7.3).
nd, not defined or undeterminable due to negative weights of dried biomass on filters.

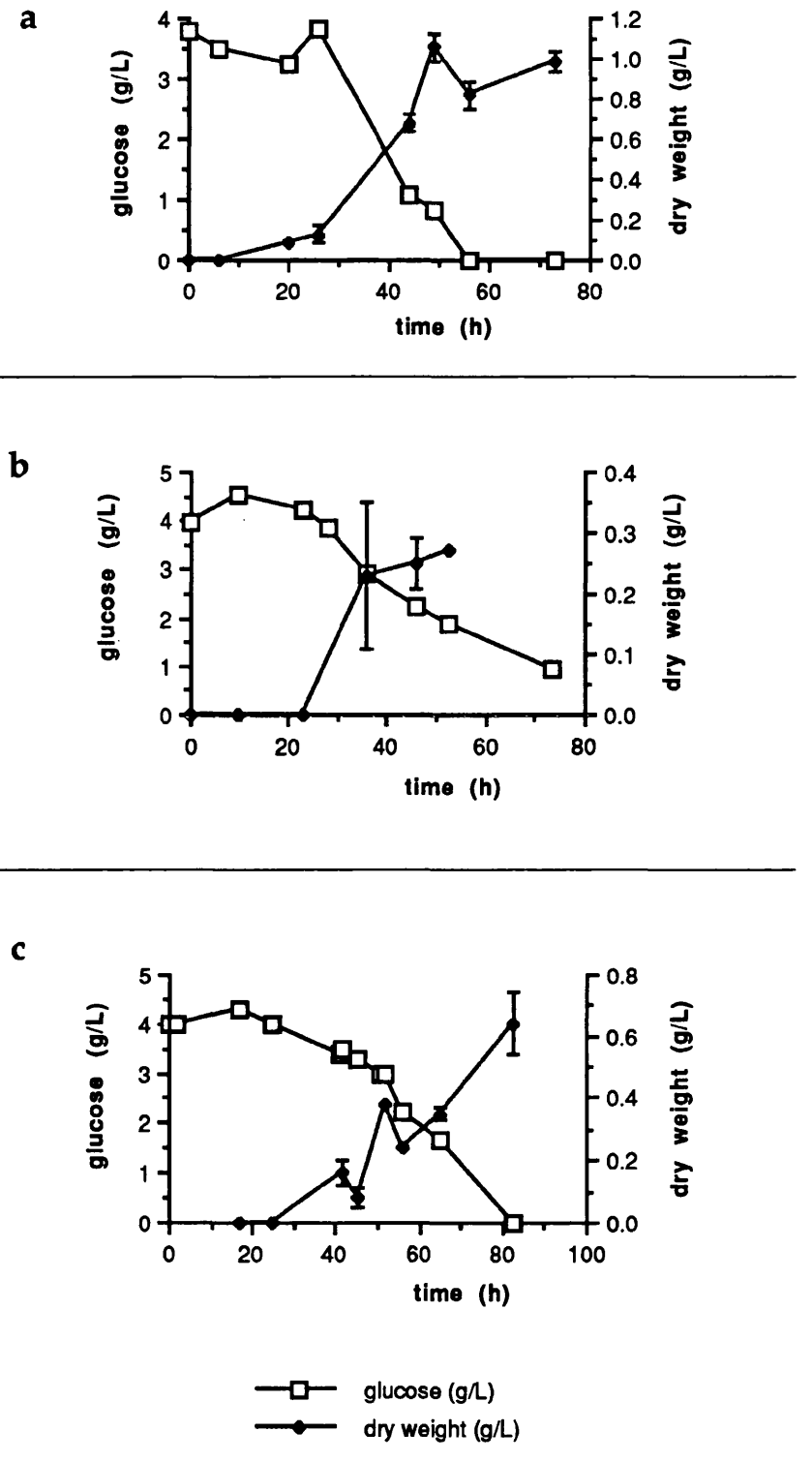


**Figure 3.7. Glucose consumption and biomass production (measured as dry weight) by *S. coelicolor* 1147 in the Bioengineering fermenter.**
Biomass samples harvested from fermentations **a**, **b** and **c** (described in figure 3.5; section 3.5.1) were measured as dry weight using the filtration method (section 2.7.2.1). Glucose was determined by the GOD PERID method (section 2.7.4.1). All values are expressed as means of triplicate measurements and standard deviations are given as error bars on the biomass curve.

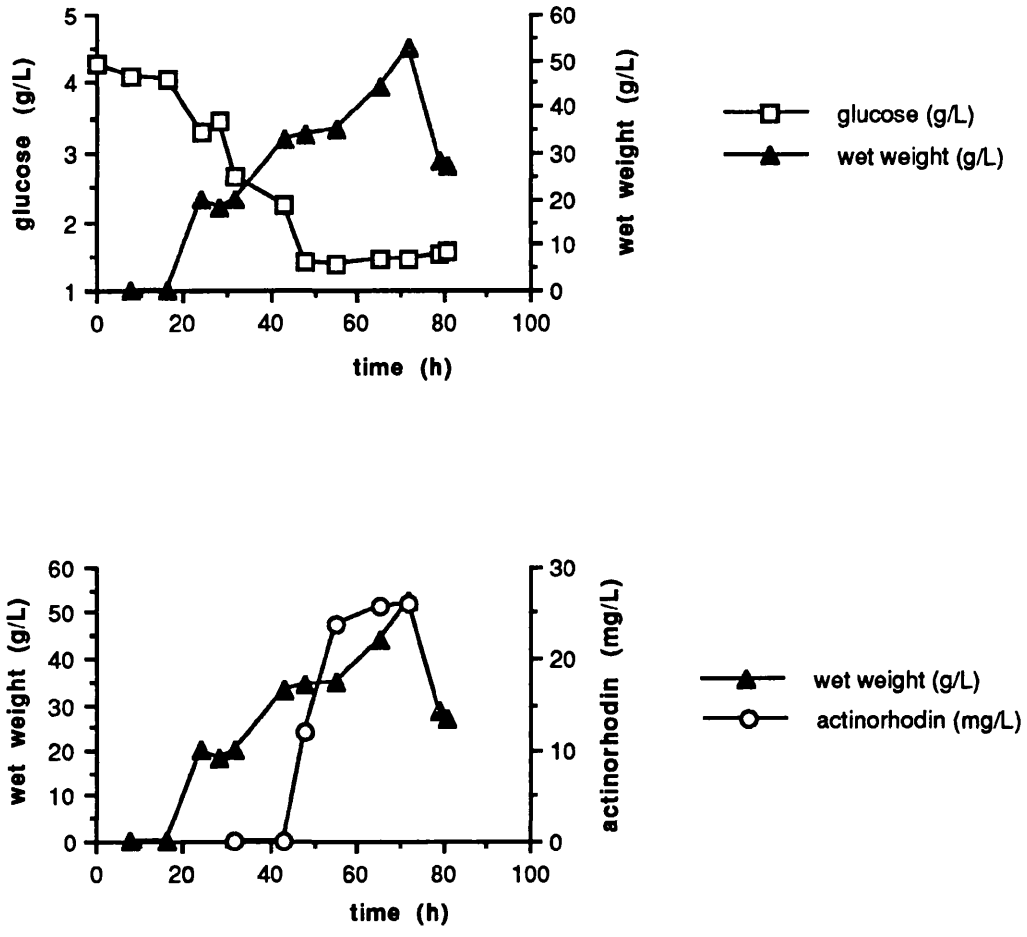


**Figure 3.8. Batch cultivation of *S. coelicolor* 1147 carried out in the Institut für Mikrobiologie und experimentelle Therapie, Jena, FRG: glucose utilization, growth and actinorhodin production.** A 3.5litre fermenter containing 3litres of NMM-J was inoculated with $1x10^7$spores.ml$^{-1}$ culture. The fermentation was run over 3 days and samples were taken on an 8 hourly basis (approximately). pH, temperature and dissolved $O_2$ were measured *in situ*, while $CO_2$ was measured in the effluent gas. Samples removed from the fermenter were assayed for glucose, using the glucose oxidase probe (section 2.7.4.2), and actinorhodin (section 2.11). Biomass wet weight measurements were determined by weighing washed biomass pellets from small culture samples (20ml; section 2.7.1); they are plotted with the curve of glucose utilization. The concentrations of biomass are also plotted with the curve of production of actinorhodin.

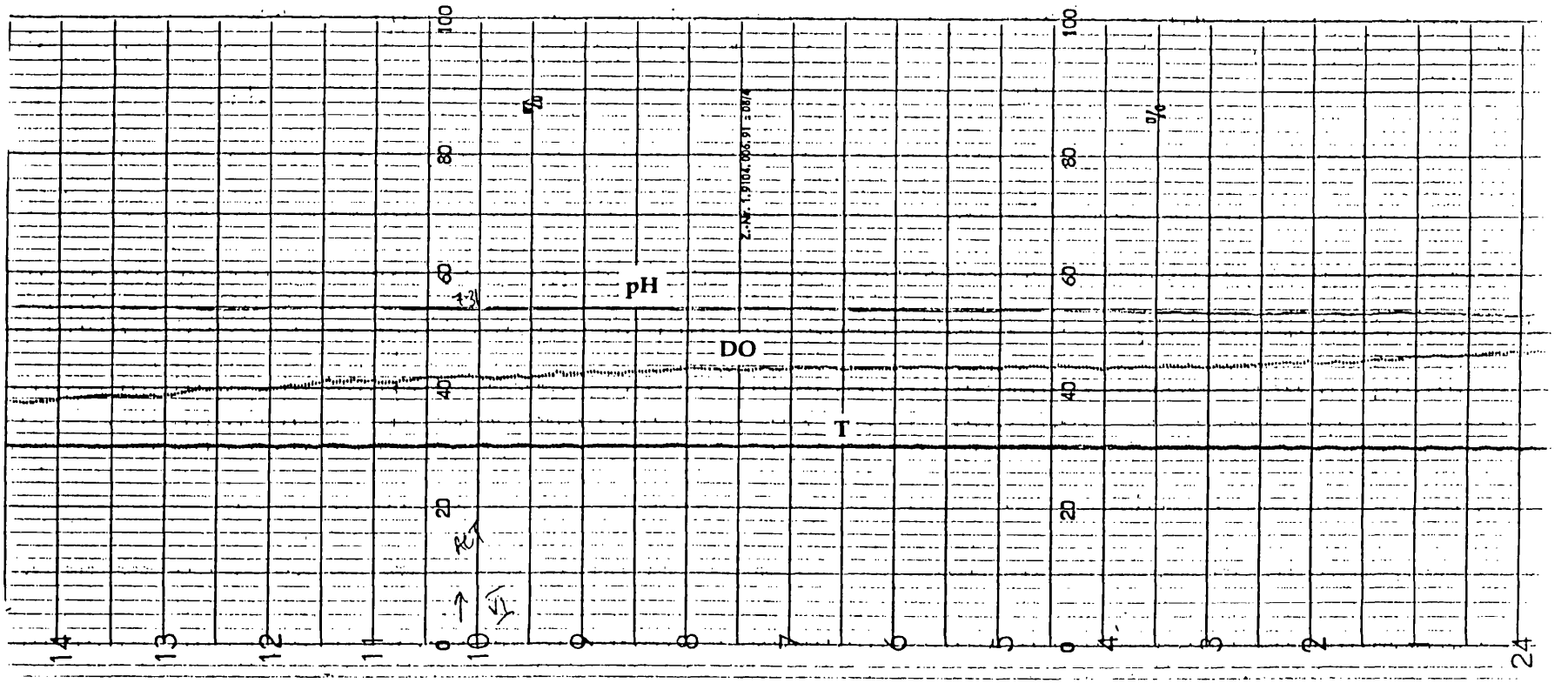


**Figure 3.9. Trace recordings of parameters measured during batch cultivation of *S. coelicolor* 1147 in Jena.** Batch cultivation of *S. coelicolor* 1147 was carried out in a 3.5litre fermenter containing 3litres of NMM-J (see figure 3.8). The fermentation was run for over three days and continuous measurements of temperature (T), pH and dissolved oxygen concentration (DO, in %) were taken. Temperature remained constant at 30°C. The horizontal axis represents time (in a 24 hour cycle) with each vertical line delineating 30 minutes.
**a.** Trace recording of pH, DO and T during the end of growth phase and at the initiation of production of actinorhodin which occurred at 43.3 hours after inoculation (as indicated by the arrow). Act, onset of production of actinorhodin; VI, sample number 6 was taken during this period.

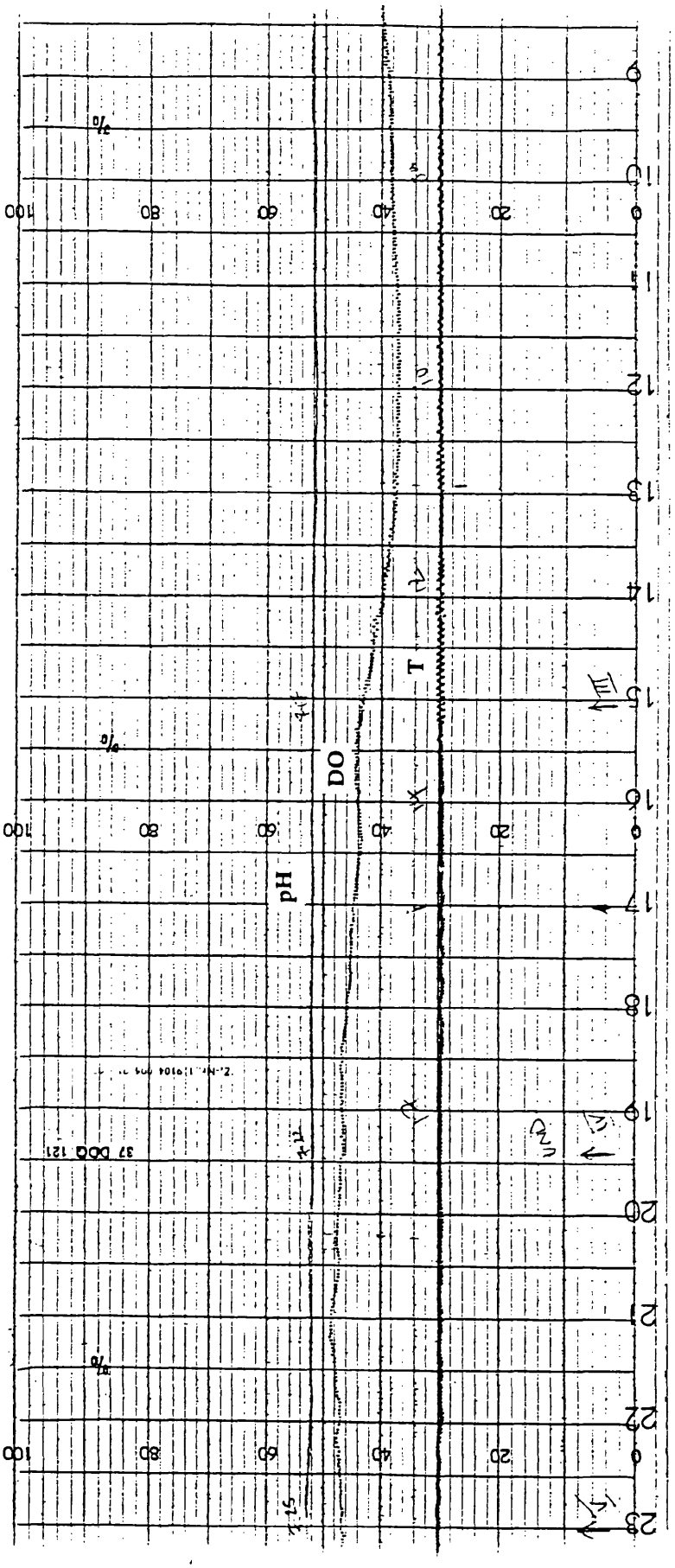


**Figure 3.9** continued. **b**. Section of trace showing changes in pH and DO recorded during growth phase and during production of undecylprodigiosin. UND, onset of production of undecylprodigiosin (28.3 hours after inoculation), as indicated by the arrow; III, IV, V, sample numbers 3, 4 and 5 were taken during this period.

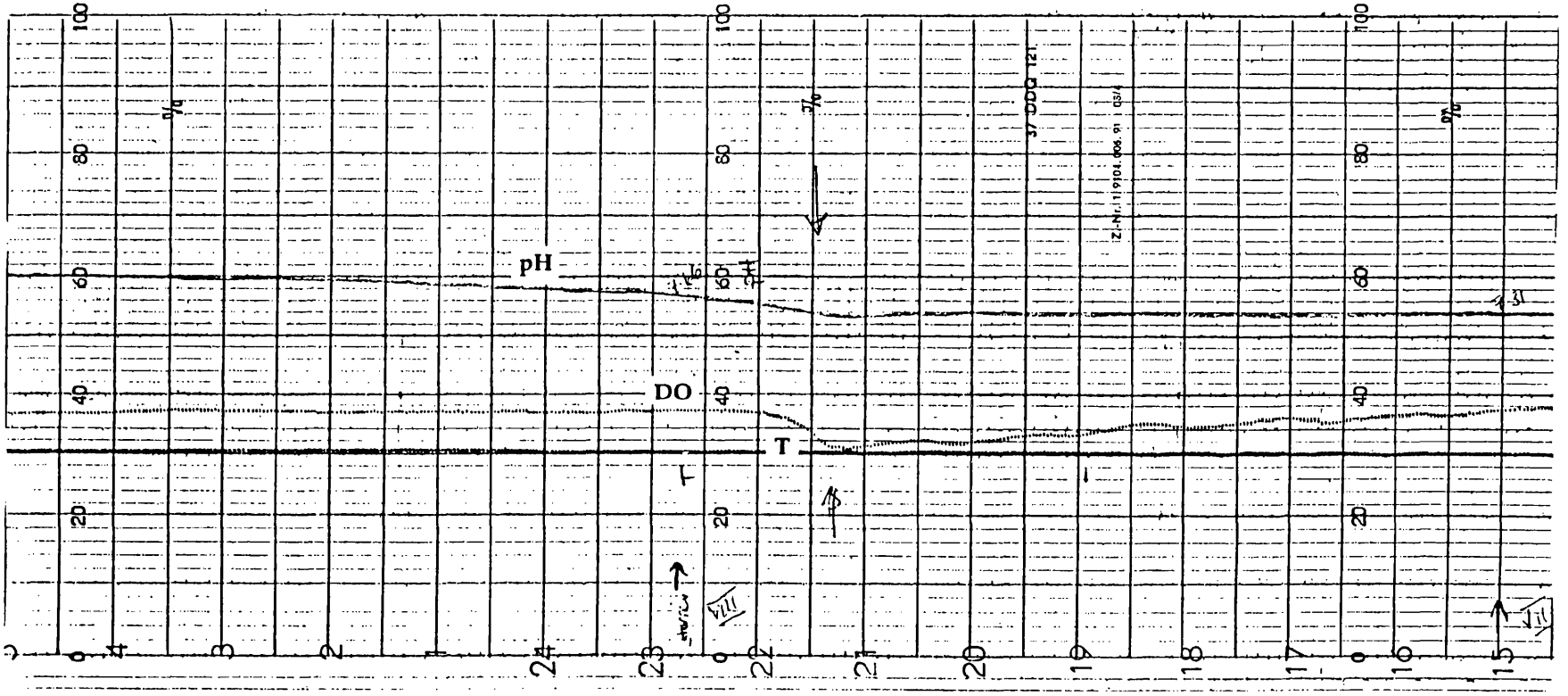


**Figure 3.9** continued. **c.** Section of trace showing changes in measured parameters during actinorhodin production. Arrows indicate the simultaneous increase in pH and DO of the culture (at 54.3 hours after inoculation). VII, VIII, sample numbers 7 and 8 were taken during this period.

**Table 3.4. Comparison of actinorhodin production by *S. coelicolor* 1147 in cultures of different phosphate concentrations.**

| TIME (h) | FLASK A | | FLASK B | |
|---|---|---|---|---|
| | dry weight ($g.l^{-1}$) | act ($mg.g^{-1}$) | dry weight ($g.l^{-1}$) | act ($mg.g^{-1}$) |
| 28.25 | 0.008 | 0.0 | 0.012 | 0.0 |
| 47.5 | 0.062 | 0.0 | 0.131 | 0.0 |
| 54.25 | 0.099 | 98.2 | 0.148 | 124.9 |
| 58.25 | 0.118 | 93.6 | 0.168 | 140.7 |
| 78.25 | 0.161 | 133.4 | 0.338 | 257.0 |
| 96.5 | 0.231 | 142.3 | 0.575 | 304.9 |
| 103.5 | 0.199 | 171.3 | 0.453 | 404.8 |

Duplicate flasks containing NMM-J and $1.5g.l^{-1}$ $K_2HPO_4$ (flask A) or $2.0g.l^{-1}$ $K_2HPO_4$ (flask B) were inoculated with *S. coelicolor* 1147 spores and shaken at 30°C. Culture samples were removed at indicated times and assayed for actinorhodin (act; section 2.11). Biomass concentrations were measured by carbon analysis (section 2.7.3).

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**Table 3.5. Final concentrations of actinorhodin produced by *S. coelicolor* when grown in differing levels of phosphate.**

| $K_2HPO_4$ ($g.l^{-1}$) | ACTINORHODIN ($mg.l^{-1}$) |
|---|---|
| 1.5 | 38.4 |
| 2.0 | 178.1 |
| 2.5 | 113.8 |
| 3.0 | 112.3 |
| 3.5 | 97.7 |
| 4.0 | 116.6 |

12 flask cultures (6 duplicates) of *S. coelicolor* 1147 were grown at 30°C in an orbital incubator from $1x10^7$ $spores.ml^{-1}$. Each set of duplicates contained a different concentration of phosphate, within a range of $1.5g.l^{-1}$ to $4.0g.l^{-1}$. The final concentrations of actinorhodin were measured (section 2.11) after four days of cultivation.

**Table 3.6. Limiting concentrations of nutrients for the continuous cultivation of *S. lividans* and *S. coelicolor*.**

**a** *S. lividans*

| LIMITATION | $NH_4Cl$ ($g.l^{-1}$) | $PO_4$ ($mg.l^{-1}$) | $KH_2PO_4$ ($mg.l^{-1}$) | GLUCOSE ($g.l^{-1}$) |
|---|---|---|---|---|
| $NH_4^+$ | 0.08 | 47.5 | 68.0 | 2.5 |
| $PO_4^{3-}$ | 0.4 | 9.5 | 13.6 | 2.5 |
| glucose | 0.4 | 47.5 | 68.0 | 0.5 |

**b** *S. coelicolor*

| LIMITATION | $NaNO_3$ ($g.l^{-1}$) | $PO_4$ ($mg.l^{-1}$) | $K_2HPO_4.3H_2O$ ($mg.l^{-1}$) | glucose ($g.l^{-1}$) |
|---|---|---|---|---|
| $NO_3^-$ | 0.486 | 190 | 458 | 6.0 |
| $PO_4^{3-}$ | 2.43 | 38 | 91.5 | 6.0 |
| glucose | 2.43 | 190 | 458 | 1.2 |

Table 3.6a shows the concentrations of nutrients used in media for continuous cultivation of *S. lividans* (M. Roth, personal communication). The limiting nutrients (C, N and P) are given in the left-hand column. Depending on the type of limitation required, the concentrations of the corresponding nutrients (the column headings) are determined from the appropriate rows. The difference between limiting and non-limiting concentrations of a nutrient is a factor of five.

Table 3.6a was used to calculate the concentration of C-, N- and P-containing compounds in the media used for continuous cultivation of *S. coelicolor;* table 3.6b. The concentrations of $NaNO_3$ and $K_2HPO_4.3H_2O$ were based on those of $NH_4^+$ and $PO_4^{3-}$ in table 3.6a which were then mulitplied by four to give higher amounts of *S. coelicolor* biomass. A concentration of $6g.l^{-1}$ glucose was found to be sufficient.

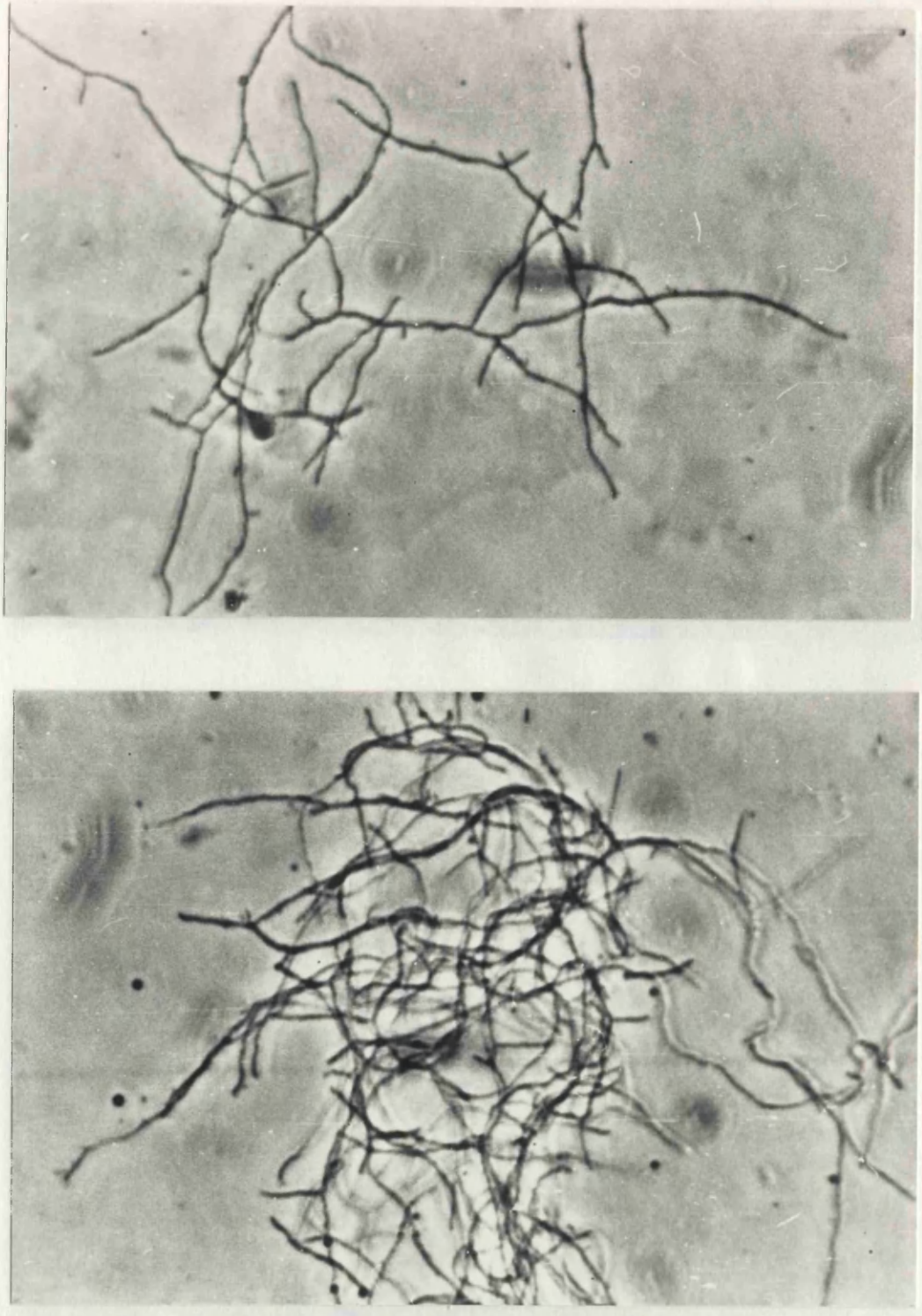


**Figure 3.10. Morphology of *S. coelicolor* mycelia at different growth rates in a phosphate-limited continuous culture.**
Continuous cultivation of *S. coelicolor* was carried out in Jena in a phosphate-limited chemostat system described in section 3.7. Samples were taken at dilution rates of $0.09h^{-1}$ and $0.12h^{-1}$. Cells were photographed using phase contrast microscopy at a magnification of X317. **a.** Morphology at $0.09h^{-1}$. Loose aggregates with long hyphae and a few short branches were visible; entangled aggregates with longer branches were also seen at this dilution rate. The mycelia were possibly as dispersed as those observed during batch growth in HMM (section 3.4).

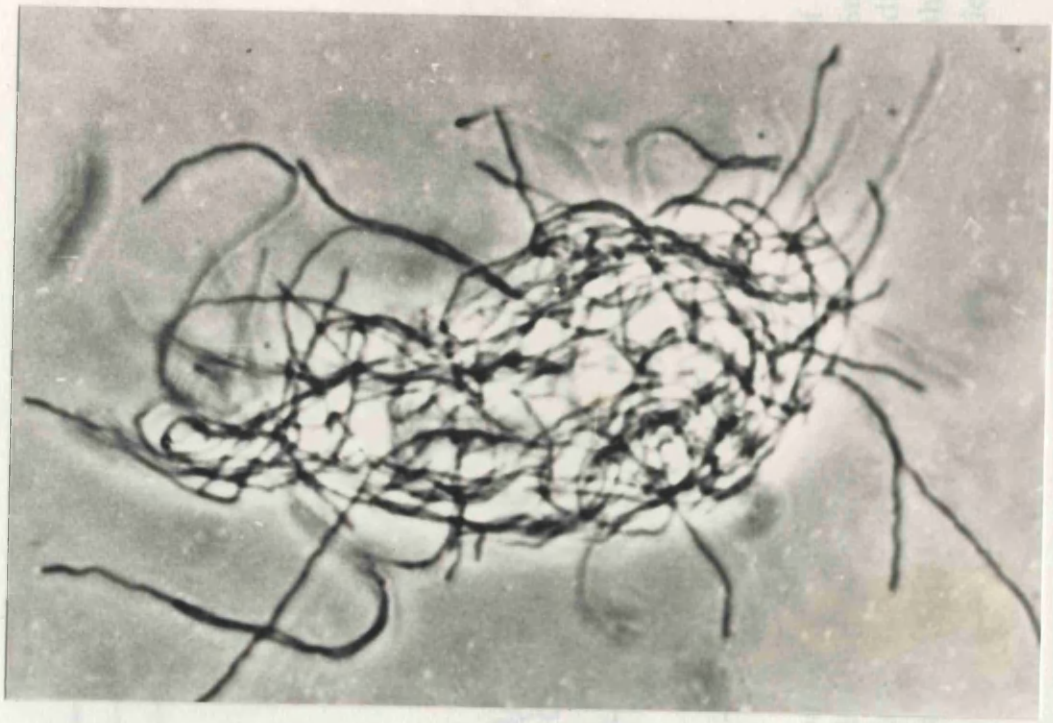


**Figure 3.10** continued. **b.** Morphology at $0.12h^{-1}$. Branches grew longer and a greater extent of entanglement occurred. However, the centres of the aggregates were not dense (*c.f.* batch culture) and smaller forms with fewer branches were also present.

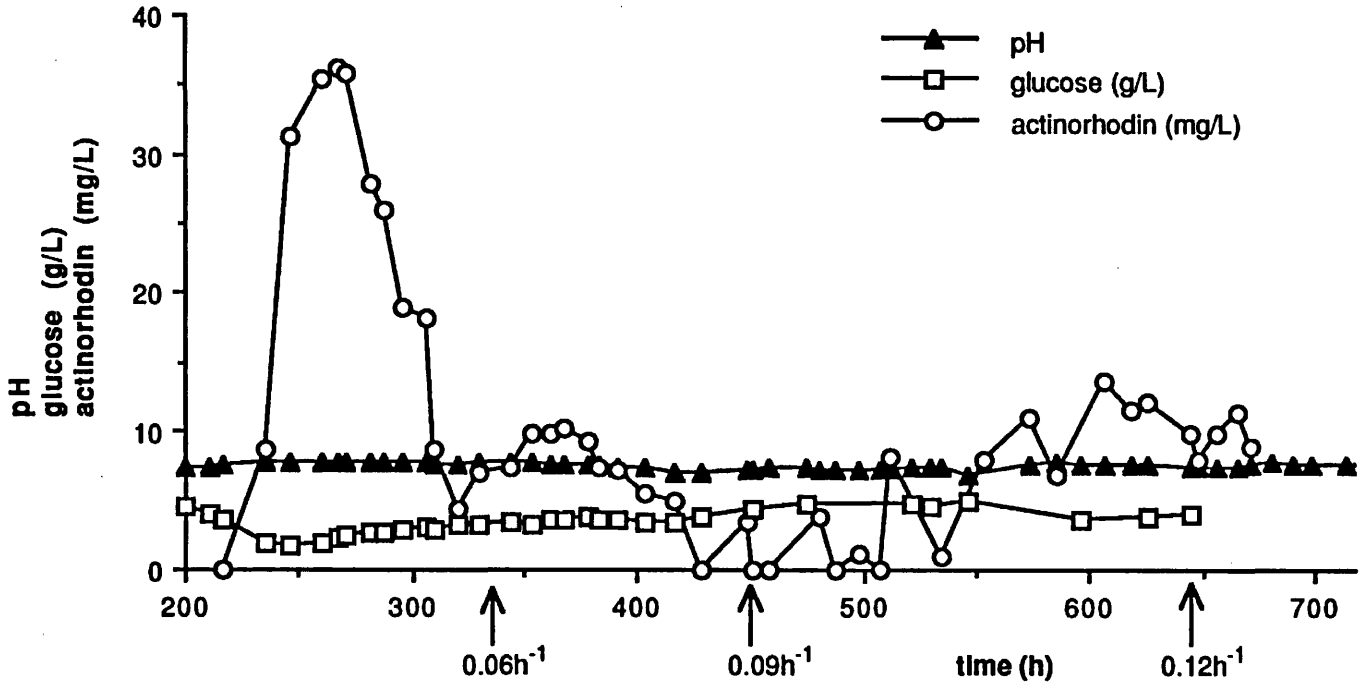



**Figure 3.11. Production of actinorhodin during continuous cultivation of *S. coelicolor* 1147 in Jena.** Continuous cultivation of *S. coelicolor* 1147 was carried out in the chemostat (section 2.6.2) containing a phosphate-limited modification of NMM-J (section 3.7.1). 6ml samples, taken every 8 hours, were assayed for residual glucose (using the glucose oxidase probe; section 2.7.4.2) and actinorhodin (section 2.11). pH measurements were taken with a chemostat pH probe (New Brunswick, USA). The initial dilution rate was $0.04h^{-1}$ which was increased to $0.06h^{-1}$, $0.09h^{-1}$ and $0.12h^{-1}$ at the indicated times (arrows). A period of five dilutions occurred at each dilution rate prior to the determination of steady-state measurements.

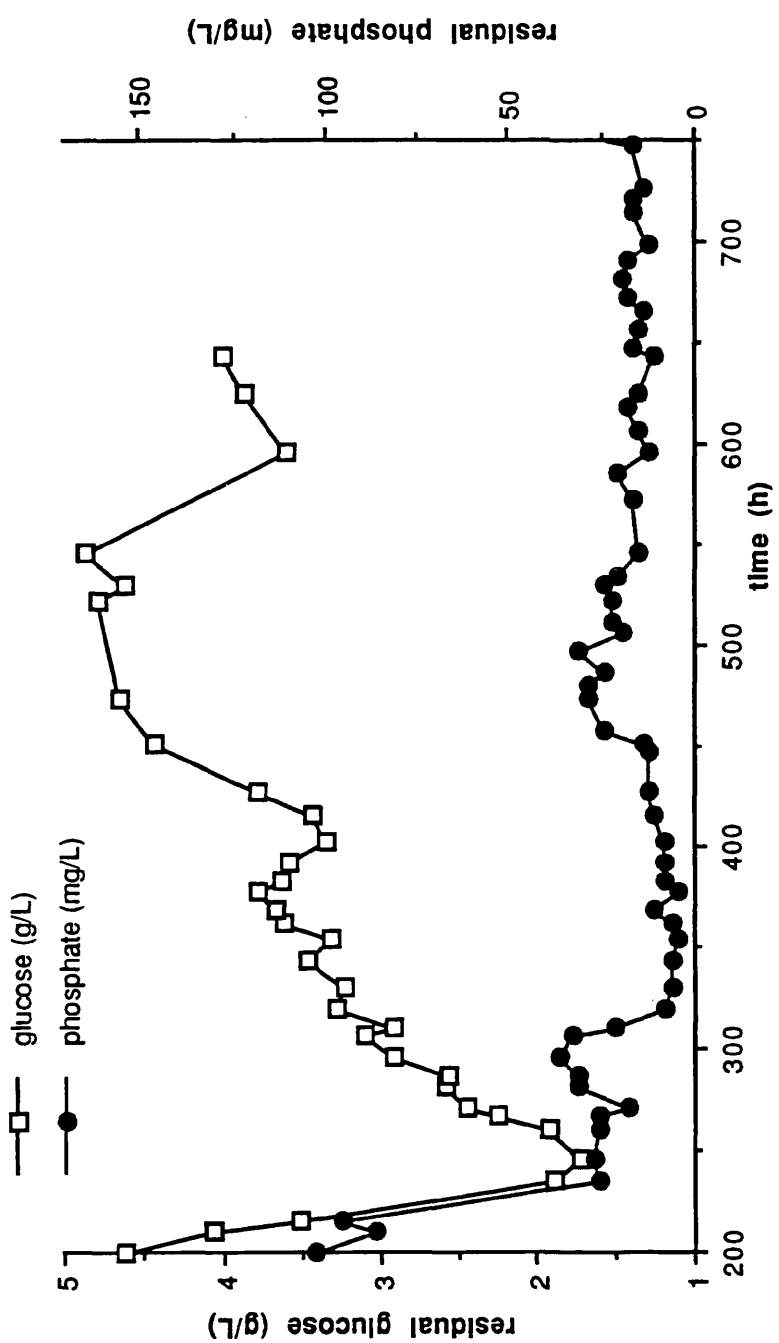



**Figure 3.12. Pattern of phosphate utilization during continuous cultivation of *S. coelicolor* 1147.** Samples taken from the continuous culture of *S. coelicolor* 1147 performed in Jena (section 3.7) were shipped to Glasgow for further analysis. Samples were subjected to phosphate (section 2.10.5) and glucose (section 2.7.4.1) determinations to show more detailed patterns of utilization. However, lysis of the samples during shipment rendered any determination doubtful. An approximation of phosphate and glucose uptake is therefore presented.

# Chapter 4

# Determination of the macromolecular, monomeric and elemental compositions of *Streptomyces coelicolor* A3(2)

## 4.1 Introduction

Calculation of the fluxes through the central metabolic pathways of *E. coli* by the method of Holms (1986) required knowledge of the monomeric composition of that bacterium. Determination of throughputs from which fluxes were calculated was based on the premise that input was equivalent to output (section 1.10). The output represented as *E. coli* biomass is composed of approximately 50% carbon, 20% oxygen, 8% hydrogen, 3% phosphorous, 1% sulphur, 2% potassium, 0.05% each of calcium, magnesium and chlorine, 0.2% iron and 0.3% trace elements (Ingraham *et al.*, 1983). At the molecular level, output is represented as large polymers (macromolecules) which are formed from low molecular weight monomers. Studies on the macromolecular content of *E. coli* (strain B) were initiated by Taylor (1946; section 1.4). The currently used composition of *E. coli* B/r, which was established by Dennis and Bremer (1974), is 55% protein, 20.5% RNA (total) and 3.1% DNA. Total RNA was determined to consist of 81.4% rRNA, 14.6% tRNA and 4.0% mRNA (Neidhardt, 1987).

Determination of the macromolecular composition was complemented by quantitation of monomers. Experiments carried out by Roberts *et al.* (1955; section 1.4) concerning the measurement of amounts of amino acids, ribonucleotides and deoxyribonucleotides in *E. coli* biomass, provided the foundation for subsequent quantitative studies. A recent report on the chemical composition of *E. coli* B/r (Neidhardt, 1987; table 4.1) was derived from this work and compiled from data of Dennis and Bremer (1974), Ingraham *et al.* (1983) and Umbarger (1977). Non-experimental data, *i.e.*, calculations based on assumptions concerning the data, were also included. An alternative composition (of *E. coli* ML308) is given in Holms (1986) which was taken from Morowitz (1968). Since the composition of a bacterium alters with $\mu$ (section 1.7.4), each set of data was accompanied by a description of the growth conditions.

Because different compositional data are available and as the latest set is a compilation of values, a chemical inventory of *E. coli* is to date incomplete (Neidhardt, 1987). However, as advances are made in the techniques used to isolate and measure cellular components, so too will increases be made in the accuracy of the composition.

## 4.2 Objectives

The ultimate aim of this thesis is to compare the fluxes through the central metabolic pathways of *S. coelicolor* to biomass with the fluxes to non-biomass associated products such as actinorhodin (section 1.11). Determination of these fluxes would be made by the Holms method (Holms 1986; section 1.10). It was therefore necessary to acquire all of the data for *S. coelicolor* biomass required to calculate these fluxes. This information is listed in the following objectives.

1. An initial aim was to determine the macromolecular composition of *S. coelicolor* 1147 during "exponential" growth phase. As stated previously (section 4.1), in glucose minimal medium *E. coli* biomass contains 55% protein, 20.5% RNA and 3.1% DNA (Dennis and Bremer, 1974; Neidhardt, 1987). The proportion of macromolecules in a bacterium depends on the growth rate of the organism (section 1.7.4). Growth differences in cultures of *S. coelicolor* (sections 3.4, 3.5) may have been attributable to differences in the growth rate, perhaps because of differences in mycelial morphology. Determination of the relative proportions of macromolecules in different cultures would allow verification of this.

Changes in macromolecular content of *S. coelicolor* have been measured at the end of vegetative growth on solid medium (Granozzi *et al.*, 1990). It was observed that protein and nucleic acid synthesis was reduced simultaneously with a cessation in growth. Growth and macromolecular synthesis resumed at the onset of differentiation. However, no such variations were observed during aerial hyphae formation in *S. alboniger* (Surowitz and Pfister, 1985b). Nevertheless, it would be of interest to determine if any changes in macromolecular composition of *S. coelicolor* occurred in liquid culture prior to antibiotic production. Knowledge of the macromolecular composition would also allow determination of some monomeric components.

2. The Holms method for calculation of fluxes (Holms, 1986; section 1.10) uses the monomeric composition of the organism in question to calculate the drain from central metabolism to biosynthesis. Therefore, the second aim of the chapter was to determine the chemical (monomeric) composition of *S. coelicolor* 1147. Monomer content may be obtained from macromolecular content. For example, approximately 70% of *Streptomyces* DNA is comprised of guanine (G) and cytosine (C) bases (Pridham and

Tresner, 1974). The DNA content may then be expressed in terms of its bases. However, not all monomer amounts can be calculated from the macromolecular composition. For example, analytical determination of amino acids was obtained by high pressure liquid chromatography (HPLC: section 2.12). An available amino acid composition of a streptomycete species would also be useful in its own right since availability of amino acids may be a consequence of codon bias.

3. The final aim of this chapter was to determine the elemental composition of *S. coelicolor*. Although this was not required for calculation of fluxes, it would give an overall view of the biomass composition. Elemental composition analyses have been reported for two other streptomycete species, *S. cattleya* (Bushell and Fryday, 1983) and *S. thermonitrificans*, a thermotolerant species (Burke, 1991). In comparison to the elemental compositions of other micro-organisms, these *Streptomyces* appeared to have a higher oxygen content. It would be of interest to compare the elemental composition of *S. coelicolor* with these other species.

## 4.3 Methodology for separation and determination of macromolecules

This section describes, in detail, why a classical method (section 2.9.1) was used to extract the different macromolecules from *S. coelicolor* biomass, and includes some modifications that were made to the colorimetric methods for macromolecular determination (section 2.10).

### 4.3.1 Separation of macromolecules

Biomass from *S. coelicolor* cultures grown in NMM-J was harvested at stated times both from flasks and from the Bioengineering fermenter. The washed pellets were subjected to fractionation method 1 (section 2.9.1) in order to separate cellular macromolecules into four fractions.

The method used for extraction of the macromolecular components of *S. coelicolor* 1147 depended on their solubility in differing acid and alkaline conditions in a four step fractionation. An initial weak acid step ( at 4°C) was required to remove sugars (including polysaccharides) and several low molecular weight molecules. These molecules included phosphorous compounds, both inorganic and low molecular weight organic, and

nucleotide co-enzymes (Hutchinson and Munro, 1961). Interference by such small molecules was therefore eliminated in subsequent fractions. Most published methods used trichloroacetic acid (TCA) for this extraction (as reviewed by Hutchinson and Munro, 1961). However, because of interference by TCA in carbon estimations of the macromolecule-containing fractions, that acid was not appropriate in this study. *S. coelicolor* biomass was, therefore, initially subjected to a cold acid step using perchloric acid (PCA, $HClO_4$).

PCA was used at concentrations of 12% (v/v; 2N) and 6% (v/v; 1N) PCA at this stage (Schneider *et al.*,1950), but according to Ogur and Rosen (1950), incubation in 6% (v/v) PCA at 4°C for 3 to 6 hours also effected RNA extraction, which was undesirable. Their method used 1.2% (v/v; 0.2N) PCA and resulted in "no detectable loss of nucleic acid from the residual pellet". A concentration of 1.2% (v/v; 0.2N) PCA at 4°C was therefore used for extraction of small molecules from *S. coelicolor* biomass.

The second step in the fractionation involved a higher concentration of PCA suitable for extraction of nucleic acids. Two consecutive 30 minute incubations of the carbohydrate-free pellet with 3% (v/v; 0.5N) PCA at 70°C (as described by Burton, 1956) allowed extraction of the RNA and 96% of the DNA. Alternative methods using lower temperatures and lower acid concentrations (Chiba and Sugahara, 1957) did not result in complete removal of DNA although RNA was fully extracted. Higher temperatures and strong acid were reported to destroy the deoxyribose groups of DNA (Hutchinson and Munro, 1961). The method of Burton (1956) was therefore used.

Extraction of protein had been described by Lowry *et al.* (1951) using 1N NaOH for 30 minutes at room temperature, or 10 minutes at 100°C. However, a much slower process of extraction was obtained by incubating the residual pellet in 0.5N NaOH at 37°C overnight.

The remaining material, both acid and alkali resistant, was resuspended in distilled $H_2O$ ($dH_2O$) and represented the fourth and final fraction. This material was assumed to be cell-wall associated, *i.e.*, peptidoglycan and protein.

### 4.3.2 Spectrophotometric determination of macromolecules

Estimations of the amount of DNA, RNA, protein and carbohydrate from *S. coelicolor* biomass and present in the fractions described previously (section 4.3.1) were carried out using the appropriate assays as described in section 2.10.

Because each macromolecule was extracted under acid or alkali conditions, standard solutions were prepared using the appropriate extraction solution as the solvent. An attempt was made to cross-check the four fractions for every macromolecule using standards dissolved in $dH_2O$. However, using standards thus prepared, a non-linear curve was obtained for DNA standards assayed by the Burton method (which reflected the requirement of PCA in the assay), whereas a lower level of absorbance was obtained from RNA standards as compared to those dissolved in 0.5N PCA. In addition, interference from carbohydrate groups in the cold PCA fraction invalidated nucleic acid estimations, while nucleic acids interfered in carbohydrate determination in the hot PCA fraction. Estimation of protein in fractions other than the alkali fraction resulted in negative absorbance measurements. Therefore, the appropriate extraction solutions were used to dissolve standards and estimation of each macromolecule in its respective fraction was carried out with these standards.

### 4.3.3 Interference by junlon

Preliminary analyses were carried out using biomass grown in HMM (section 2.5.1), *i.e.*, minimal medium containing junlon. Because junlon is difficult to remove from mycelia (sections 3.2, 3.3), in addition to complicating carbon analyses, it was possible that it would interfere with some of the spectrophotometric assays. Junlon is a polyacrylate and could, therefore, be detected by the carbohydrate assay and would possibly interfere with the RNA assay.

An attempt was made to establish the extent of interference, if any, by junlon in the orcinol (section 2.10.3) and anthrone (section 2.10.1) assays. Analyses were performed on equal volumes of samples containing either biomass plus junlon or junlon alone. These samples had been "harvested" from an *S. coelicolor* culture and from an equivalent volume of HMM. Junlon did not appear to react with the reagents for DNA and protein

determination, but reacted positively with the orcinol and anthrone reagents (table 4.2; a and b, respectively). Quantitation of junlon was not possible (since it is neither an RNA nor a carbohydrate molecule) but an absorption comparison could be made. When assayed by the anthrone method, junlon gave an absorbance value 1.4 fold greater than the biomass plus junlon. Contrary to this, on assaying by the orcinol method, the absorption value of junlon was less than that of biomass plus junlon. However, junlon visibly interfered with the reagent causing the final product to be an orange colour indicating a shift in the maximum absorption wavelength. An orange colour had been produced during the reaction of the orcinol reagent with hexose molecules (Brown, 1946).

Alterations in absorbance wavelength of the final product obtained by the reaction of junlon with the orcinol reagent were examined further by recording the absorbance spectrum of the chromophore in the reaction from 400nm to 700nm. To achieve this, three substrates were prepared for the orcinol assay and measured against the appropriate blanks:

a) 150µg RNA (standard)
b) 1.5ml of junlon (10% (w/v) wet junlon obtained by centrifugation of HMM)
c) 150µg RNA plus 1.5ml of 10% (w/v) junlon.

Products of the assay were scanned between 400nm and 700nm. The RNA standard gave an absorbance peak (0.72) at approximately 665nm with a sample blank of 0.5N PCA (figure 4.1a). The scan of the product of the orcinol reaction in the presence of junlon alone (which was measured against an $H_2O$ blank) had a maximum absorbance wavelength at approximately 490nm (figure 4.1b). Junlon also gave a high absorbance at 665nm (1.26) but this may have been due to the relatively high concentration of junlon. Addition of 150µg of RNA to the junlon sample also resulted in two absorbance peaks, at 665nm and 490nm (figure 4.2c); at 665nm the absorbance value was 1.75. The difference in the absorbance value of RNA caused by the presence of junlon (1.03) did not correspond to the absorbance of junlon alone at 665nm (1.26). Thus a quenching effect was caused by junlon.

A similar experiment was performed using the anthrone assay and a glucose standard of 100µg. A scan of the standard product showed an absorbance maximum (0.82) at 620nm (figure 4.2a). An additional peak was

observed at approximately 510nm (0.52). Junlon alone gave a peak at 620nm (figure 4.2b) with an absorbance value of 0.33. Additional peaks included one at 435nm (0.39) and a shoulder at 510nm (0.225). Addition of 100µg of glucose to the junlon sample increased the absorption values of all three peaks. The peak at 620nm exhibited the absorbance maximum (1.13; figure 4.2c). Subtraction of the junlon absorbance value at 620nm (0.33) from that of junlon plus glucose at the same wavelength (1.13) gave an absorbance equivalent to that of the glucose standard (0.8). Applying the same process to the peak at 510nm gave the same result. Therefore, junlon was observed to have an additive effect on the anthrone method. However, since it was not possible to quantify the junlon, a factor could not be introduced to take account of the additive interference.

The extent of interference by junlon in the two methods, and the difficulty in determining carbon content of the biomass, led to the removal of junlon from HMM and the investigation of alternative dispersal methods (section 3.3).

## 4.4 Macromolecular composition of flask grown *S. coelicolor* 1147 biomass

The initial aim of this chapter was to determine the macromolecular composition of *S. coelicolor* (section 4.2). The methods for extraction and estimation of protein, RNA and DNA, (sections 2.9.1, 2.10) were used to analyse *S. coelicolor* 1147 biomass which had been grown in 50ml of NMM-J (section 2.5.1).

### 4.4.1 Preliminary analysis for estimation of macromolecular content.

Prior to determining the composition of *S. coelicolor* 1147 biomass, it was necessary to establish the reproducibility of the fractionation method (section 2.9.1) and that of the spectrophotometric estimation methods (section 2.10). Three biomass samples (1, 2 and 3) were used to ascertain the reproducibilities. To obtain sufficient biomass for analysis, each biomass sample was the product of pooling two or three cultures of *S. coelicolor* 1147. Each sample was divided into two (a and b) and every portion was subjected to fractionation method 1 at a concentration of 100mg (wet weight).$ml^{-1}$. The resulting fractions were then assayed for macromolecular

content and the estimated macromolecules were expressed as mg per g wet weight (table 4.3). Samples 1a and 1b were analyzed for each macromolecule and also carbohydrate content. The close values indicated reproducibility within the fractionation and measurement methods. The consistency of the fractionation method and the orcinol and Burton methods were verified by samples 2 and 3.

These methods for determining nucleic acids plus the anthrone and Bradford methods were therefore used to determine the macromolecular content of *S. coelicolor* grown in flasks. In total, 53 flask cultures were used, several of which were harvested at similar times to allow statistical analysis. From these cultures, some of which were pooled, 23 biomass samples were obtained which underwent fractionation and macromolecular measurements. The macromolecular composition of each sample was calculated in terms of dry weight ($mg.g^{-1}$ dry weight, as estimated by carbon analysis; section 2.7.3) and contents from samples harvested at similar times were expressed as means (table 4.4). Standard deviations, which are distribution parameters, were also calculated to give an indication of the extent of distribution of the values about the means (Green and Margerison, 1978); these are expressed in the same units as the means.

The size of the standard deviations from the means in table 4.4 (from 2% to 90% of the mean values) indicated that large differences existed between similarly grown biomass samples. Pooling of cultures had not appeared to even out these differences. A complementary experiment using *S. coelicolor* 209 had shown that there were differences in macromolecular compositions of samples harvested at the same time (section 3.3.2). However, the differences were not as great as those when macromolecular contents were grouped in terms of progression of the fermentation and of antibiotic production. Table 4.4 was constructed using *S. coelicolor* 1147 biomass harvested at similar times irrespective of antibiotic production. Alternative statistical analysis was therefore carried out using the same biomass but grouped with respect to extent of antibiotic production (table 4.5). In this case, the standard deviations were in the same range as those shown in table 4.4. Therefore, it was not possible to suggest whether time of harvesting or antibiotic production could be used as an indication of biomass samples having similar macromolecular contents.

The overall picture of macromolecular composition of *S. coelicolor* 1147 samples given in tables 4.4 and 4.5 is one of great variation. Nevertheless,

use of all the mean values given in table 4.4 allowed a tentative composition of *S. coelicolor* 1147 to be given as 10% - 15% RNA, 4% - 6% DNA, 12% - 21% protein with 12% - 25% carbohydrate. A similar rough estimation of composition was obtained using table 4.5 and the percentages agreed with those obtained for *S. coelicolor* 209 (section 3.3.2), although the protein estimate was slightly lower for *S. coelicolor* 1147. The macromolecular composition of *E. coli* grown in a glucose minimal medium is 55% protein, 20.5% RNA and 3.1% DNA (section 4.1). However, the macromolecular composition of *E. coli* changes with growth rate (section 1.5.4). In this medium, *E. coli* has a doubling time of 40 minutes whereas *S. coelicolor* grows in NMM-J with an approximate doubling time of 8 hours. It was therefore more feasible to compare the composition of *S. coelicolor* with the composition of *E. coli* growing at a slower growth rate. At a doubling time of 100 minutes, *E. coli* has the composition: 72% protein, 14% RNA and 5% DNA. The nucleic acid contents of both species at these slow growth rates were therefore very similar, although, in absolute terms, the *S. coelicolor* chromosome (at 8megabases (Mb); Kieser *et al.*, 1992) is 75% larger than that of *E. coli* (4.6 to 4.7Mb; Smith *et al.*, 1987).

The main apparent difference in macromolecular composition of *S. coelicolor* and *E. coli*, however, was the protein content. *E. coli* contains 55% protein (or 72%, at low growth rates). According to the above results, *S. coelicolor* was measured to have up to 21% protein. This suggests that *S. coelicolor* either contains only 21% protein or that an error had occurred in the estimation of protein (the protein results were obtained using the Bradford assay; section 2.10.4.1). To determine if an error in measurement of protein had occurred, several investigations were carried out. These included determination of the carbon content of each biomass fraction with which the amount of carbon derived from the macromolecule(s) in each fraction could be compared. Alternative methods of protein estimation were also used.

### 4.4.2 Carbon analysis of biomass fractions

Determination of carbon concentration in biomass fractions (by the TOCA; section 2.7.3) allowed the relative amount of biomass carbon in each fraction to be calculated. Comparison of the carbon content of the fractions with the amount of carbon derived from macromolecules in each fraction also aided in determining the presence of previously undetected molecules.

All fractions of biomass samples described previously in this section (4.4) were analyzed for carbon content using the TOCA (section 2.7.3). To determine the proportion of biomass carbon extracted into each fraction, the values were expressed as a percentage of the total carbon content of the biomass (table 4.6). Generally, the alkali fraction contained the largest portion of the biomass carbon with values of greater than 50%. The hot PCA fraction contained 17% - 24% of the total carbon, which agreed with the estimated combined nucleic acid content, while the cold PCA had extracted the least carbon. It was expected that the cold PCA fraction would comprise a minor fraction as only small molecules such as nucleotides and amino acids would have been solubilized by the weak acid (section 4.3.1). The final fraction of *S. coelicolor* biomass (the residue fraction) contained approximately 20% of the total carbon of the biomass. Cell wall associated material was present within this fraction (section 4.3.1). Because *Streptomyces* are Gram-positive bacteria and therefore have extensive cell walls, it seemed feasible that a large proportion of the biomass carbon was present in the residue fraction. Therefore, the proportion of biomass carbon extracted into each fraction appeared feasible.

The alkali fraction was expected to contain only protein (ID Hamilton and D Mousdale, personal communication). On this assumption, the amount of total carbon extracted into the alkali fraction (approximately 50% to 56%) was comparable with the reported proportion of *E. coli* biomass measured as protein (55%: section 4.1). However, the values of carbon corresponding to *S. coelicolor* protein did not agree with the measured values of protein (21%). This suggested that only a proportion of the protein present within the alkali fractions had been detected by the Bradford assay. To verify this and also to check the content of all the biomass fractions, a comparison was made between the carbon contained in each fraction and the carbon represented by each measured macromolecule. To express these macromolecules in terms of carbon, the following equations were used:

a) <u>CHO carbon</u>: $\mu g\ CHO.ml^{-1} \times 0.4 = ppmC\ (\mu g.ml^{-1})$,

assuming CHO (measured as glucose) has a molecular weight of 180 with an average of 6 carbon atoms per molecule.

b) protein carbon: $\mu g\ protein.ml^{-1} \times 0.5 = ppmC\ (\mu g.ml^{-1})$,

assuming each amino acid has an average molecular weight of 120 with 5 carbon atoms per molecule.

c) RNA carbon:

$$\frac{\mu gRNA.ml^{-1}}{340} = \mu moleRNA.ml^{-1}$$

$$\begin{aligned} \mu mole\ RNA.ml^{-1} \times 0.218 &= \mu mole\ AMP.ml^{-1} \\ \times 0.192 &= \mu mole\ UMP.ml^{-1} \\ \times 0.329 &= \mu mole\ GMP.ml^{-1} \\ \times 0.263 &= \mu mole\ CMP.ml^{-1}, \end{aligned}$$

assuming RNA is composed of 21.8% A, 19.2% U, 32.9% G and 26.3% C (see Appendix B).

$$\begin{aligned} &\mu mole.ml^{-1}\ AMP \times 10 \\ +\ &\mu mole.ml^{-1}\ UMP \times 9 \\ +\ &\mu mole.ml^{-1}\ GMP \times 10 \\ +\ &\mu mole.ml^{-1}\ CMP \times 9 = total\ \mu mole.ml^{-1}\ C \end{aligned}$$

$$total\ \mu mole.ml^{-1}\ C \times 12 = \mu gC.ml^{-1}\ (ppm).$$

d) DNA carbon:

$$\frac{\mu gDNA.ml^{-1}}{324} = \mu moleDNA.ml^{-1}$$

$$\begin{aligned} \mu mole\ DNA.ml^{-1} \times 0.15 &= \mu mole\ AMP.ml^{-1} \\ \times 0.15 &= \mu mole\ TMP.ml^{-1} \\ \times 0.35 &= \mu mole\ GMP.ml^{-1} \\ \times 0.35 &= \mu mole\ CMP.ml^{-1}, \end{aligned}$$

assuming *Streptomyces* DNA contains 70% GC mol.

$$\begin{aligned} &\mu mole.ml^{-1}\ AMP \times 10 \\ +\ &\mu mole.ml^{-1}\ TMP \times 9 \\ +\ &\mu mole.ml^{-1}\ GMP \times 10 \\ +\ &\mu mole.ml^{-1}\ CMP \times 9 = total\ \mu mole.ml^{-1}\ C \end{aligned}$$

$$total\ \mu mole.ml^{-1}\ C \times 12 = \mu gC.ml^{-1}\ (ppm).$$

The calculated carbon values from the macromolecular content of each fraction and the total carbon concentration in the fractions were tabulated to allow comparison (table 4.7).

Expression of the carbon derived from measured carbohydrate values (CHO) as a percentage of the total carbon in the cold PCA fraction (COLD) revealed that carbohydrate represented up to 30% (and as little as 5%) of the total carbon. The remaining carbon in the cold PCA fraction probably comprised amino acids and small phosphate-containing molecules (section 4.3.1). Subsequent determination of phosphate in this fraction (section 4.5.2) showed that this was probably the case. The combined concentrations of carbon derived from the nucleic acids (RNA and DNA) also did not comprise the total carbon present in the hot PCA fraction (HOT). Up to 70% of the carbon in the hot PCA fraction of most samples was accounted for. However, the combined nucleic acid values exceeded that of the total carbon in the hot PCA fraction of sample B. Therefore, total carbon in this fraction was low relative to that in the fractions from the other samples although the RNA and DNA values were comparable. The remaining carbon suggested that either incomplete estimation of the nucleic acid content of the hot PCA fractions had occurred or that an additional acid-soluble compound had been extracted into the fractions. Because the values of nucleic acids expressed as per gram of biomass (dry weight) were feasible, it would appear unlikely that the excess carbon was due to undetermined nucleic acid. Therefore, the additional carbon was likely to be derived from an alternative molecule. A possible suggestion was the cell-wall associated polymer, teichoic acid. Attempts made to characterize and estimate the quantity of this unknown molecule are described in section 4.5.3.

Total carbon in the protein- (PROT) containing alkali and residue fractions (ALK and RES) was also only partially accounted for. The residue fraction was assumed to contain peptidoglycan in addition to cell-wall associated proteins. Therefore, carbon derived from protein was not expected to be equal to the total carbon present in the fraction. However, on expressing the carbon derived from the protein in the alkali fraction (as measured by the Bradford assay; section 2.10.4.1) as a percentage of the total carbon present, less than 50% of the total carbon was identified. Three questions were therefore raised:

1) did protein contribute to all the carbon present in the alkali fraction but had not been fully detected by the Bradford assay? or

2) had inefficient extraction of protein into the alkali fraction occurred resulting in residual soluble protein in the residue fraction? or
3) had protein degradation occurred during fractionation of the biomass resulting in extraction of amino acids and small peptides into the preceding acid fractions?
An attempt was made to examine these questions in the following sections.

### 4.4.3 Protein determination and extraction - an investigation

Since a standard protein assay (Bradford assay; section 2.10.4.1) could only detect up to 50% of the carbon present in the alkali fraction (which was assumed to contain solely protein; section 4.4.2), an attempt was made to fully quantify the protein using alternative means of determination.

Initially, the Lowry method for protein determination (section 2.10.4.2) was used. However, this assay is sensitive to interference from a large number of chemicals including NaOH (Smith *et al.*, 1985). Protein from *S. coelicolor* biomass was extracted into 0.5N NaOH during fractionation. Consequently, BSA standards were dissolved in the same solvent but were shown to have lower absorbance equivalents than standards dissolved in $H_2O$. Although all protein determinations carried out using the Lowry assay included BSA standards dissolved in 0.5N NaOH, measurement of protein from three different biomass samples using this assay accounted for less than 30% of the dry weight (table 4.8a). The measured contents of two samples (21.7% and 26.2%) were greater than those determined by the Bradford assay (approximately 21%; section 4.4.1), but did not account for all the carbon in the alkali fraction. A further attempt was therefore made to measure the protein content of *S. coelicolor* using the BCA method (section 2.10.4.3).

The method using bicinchoninic acid requires a very high pH (pH 11.25) and the assay is resistant to interference from a variety of chemicals including 0.1N NaOH (section 2.10.4.3). Therefore, standards dissolved in $H_2O$ were used to determine the amount of protein in the alkali fractions of three different samples of *S. coelicolor* biomass. The Bradford assay was also used to allow comparison of the two methods. The level of protein detection by the BCA assay was comparable to, but slightly lower than, that by the Bradford assay (table 4.8b). Therefore, the BCA method also seemed unsuitable for protein estimation in 0.5N NaOH. The use of standards dissolved in 0.5N NaOH resulted in a purple colour which was darker than

that observed using standards in $H_2O$. Interference by 0.5N NaOH was therefore visible.

All the above methods for protein estimation use whole proteins or peptide chains as substrates but are unlikely to detect amino acids (section 2.10.4). Inefficient detection of protein from *S. coelicolor* biomass in 0.5N NaOH thus suggested the possibility that protein degradation had occurred during the alkali extraction. This would have resulted in the presence of amino acids or peptide chains too small to be measured. An attempt was therefore made to establish the presence of single amino acids in the alkali fraction which could possibly account for the remaining 60% of the measured carbon. This involved use of the Ninhydrin assay during which a reaction occurs between the reagent and free amino groups (section 2.10.4.4).

Alkali fractions of several of the samples described in tables 4.5 to 4.7 were subjected to an alkali digestion step in 13.5N NaOH to liberate free amino acids (section 2.10.4.4). The resulting amino groups were quantified using the Ninhydrin assay (table 4.9, ). A comparison was then made with the amount of carbon estimated from the measured amino acid content (column II) and the amount of carbon detected by the TOCA (section 2.7.3; column I ) in these samples. However, the Ninhydrin assay did not detect all of the carbon (column III). Nevertheless, the amount of protein estimated by the Ninhydrin assay was generally greater than that measured by the Bradford assay (section 4.4.1).

To ascertain the possibility that the higher percentage of carbon accounted for by the Ninhydrin assay was due to the ability to detect free amino acids rather than proteins, the alkali fractions were again assayed by this method but the digestion step using 13.5N NaOH was eliminated. Because the assay detects free amino groups, only those of single amino acids and amino acids at the N-terminal of proteins would be measured. Subtraction of this measurement from the previous one involving liberated amino acids would give an indication of the amount of protein or peptide chains present within the fraction. Therefore, subtraction of the results of this assay (table 4.9, columnIV) from the previous assay (column II) gave the concentration of undetected proteins (column V). The concentrations were then expressed in terms of carbon which could be compared with the methods used for measurement of proteins (Bradford, Lowry and BCA). The percentage of total carbon derived from these calculations (columnVI) only corresponded to that measured by the Bradford assay in the second set of samples. The

differences in the other samples may be attributable to protein degradation within the alkali fraction between times of assaying by the Bradford and Ninhydrin assays, sensitivity of the methods or number of cultures used in the calculations.

The percentage of carbon in the alkali fraction measured by the Ninhydrin assay (including digestion in 13.5N NaOH) was surprisingly low since it was expected that the carbon in the fraction was derived from both proteins and amino acids. An experiment was therefore carried out to determine the reason for the inefficiency of this method. Firstly, the effect of the digestion step (using 13.5N NaOH) on the leucine standard was investigated. This was performed by comparing three standards (**a**, **b** and **c**) assayed by the Ninhydrin method in triplicate:

**a**) 1mM leucine - 500µl
**b**) 1mM leucine digested - 500µl (*i.e.*, 100µl of 30mM leucine digested with 900µl 13.5N NaOH, then neutralized using 2ml of acetic acid )
**c**) 1mM leucine diluted from 30mM leucine (*i.e.*, 100µl 30mM leucine plus 900µl $H_2O$ plus 2ml $H_2O$).

Standard **a** was used as the normal standard by which to determine the concentrations of standards **b** and **c** using the Ninhydrin assay. Relative to standard **a**, standard **b** was measured as 28.2mM amino groups and standard **c** was measured as 29.6mM amino groups. The inclusion of a digestion step using 13.5N NaOH therefore resulted in slight reduction of the amino group concentration. However, the concentration of standard **b** was within a 10% error limit of standard **c** suggesting that, by taking experimental error into consideration, the digestion step did not interfere with amino group measurement. It is also not likely that the concentration of NaOH in the alkali fraction (0.5N) interfered with the assay as the digestion step required a much higher concentration of NaOH.

Subsequent investigations focussed on the extraction method from which the alkali fraction arose (section 2.9.1). The alkali fraction had been previously determined to contain only protein (ID Hamilton and D Mousedale, personal communication) but in this study the result was verified by carrying out the fractionation using a BSA standard in combination with standards of RNA and DNA. Pellets of *S. coelicolor* biomass at a concentration of 100mg (wet weight). $ml^{-1}$ had been used previously for fractionation method 1 (section 2.9.1). Corresponding BSA,

RNA and DNA concentrations were therefore used in this experiment. These were 2.5mg.ml$^{-1}$ BSA, 0.75mg.ml$^{-1}$ RNA and 0.25mg.ml$^{-1}$ DNA, based on the assumption that 5% of wet weight was dry weight (section 3.5.1), 50% of dry weight was protein, 15% of dry weight was RNA and 5% of dry weight was DNA (section 4.4.1).

A preliminary experiment was carried out where 2.5mg.ml$^{-1}$ of BSA was subjected to fractionation method 1. The amino group concentration of each resulting fraction was determined by the Ninhydrin assay (section 2.10.4.4) in duplicate (table 4.10a, column A) and then expressed as μg.ml$^{-1}$ of protein (column B). The majority of the BSA was detected in the alkali fraction (92%) with a very small percentage in the cold PCA fraction (2.3%) and the remainder in the hot PCA fraction (5.6%). No ninhydrin reacting material was present in the residue fraction. However, the total concentration of BSA detected was equivalent to 1.69mg.ml$^{-1}$ of starting material whereas the starting material had been 2.5mg.ml$^{-1}$. To check that the original BSA standard solution (10mg.ml$^{-1}$; which had been diluted to provide the 2.5mg.ml$^{-1}$ solution) was at the correct concentration, it was subjected to the Ninhydrin assay. The concentration of BSA measured was 6.44mg.ml$^{-1}$. To verify this measurement, the 10mg.ml$^{-1}$ BSA solution was then checked using carbon analysis (section 2.7.3). The amount of total organic carbon determined in the solution was 4746.3ppm. This corresponded to 9.5mg.ml$^{-1}$ BSA. The ninhydrin reagent had therefore detected only 68% of the BSA in the solution. Subsequent carbon analysis of the fractions containing BSA (table 4.10a, column C) showed that the cold PCA, hot PCA and alkali fractions contained 2.6%, 1.8% and 93.8% of the carbon respectively. The residue fraction also contained carbon (1.8%). Expression of the carbon results as protein concentrations (column D) resulted in values which did not correspond to the measured values (column B). Total protein determined by carbon analysis was 2.69mg.ml$^{-1}$ which was closer to the expected concentration of 2.5mg.ml$^{-1}$. It was evident therefore that, although the majority of the BSA had been extracted into the alkali fraction (92% to 94%) with minor retention in the cold and hot PCA fractions, the Ninhydrin assay had detected only 62% (approximately) of the BSA in the fractions.

Estimation of protein from *S. coelicolor* biomass by the Ninhydrin assay had resulted in the detection of, on average, 50% to 60% of the carbon in the alkali fraction. To determine the effect of the presence of additional molecules on the extraction of protein, if any, RNA and DNA standards

were combined with a BSA standard to give concentrations stated above (*i.e.*, 2.5mg.ml$^{-1}$ BSA, 0.75mg.ml$^{-1}$ RNA and 0.25mg.ml$^{-1}$ DNA). The combined solution was then subjected to fractionation method 1 (section 2.9.1). The cold PCA fraction, hot PCA fraction and alkali fraction again reacted positively with the ninhydrin reagent (table 4.10b, column A). It was not possible to determine the exact concentration of BSA in the acid fractions, however, because extraction of RNA alone and DNA alone (which contain amino groups) also resulted in the presence of ninhydrin reacting material in the cold and hot PCA fractions (table 4.10c), although the signal was less than 10% of the total concentration in these fractions. However, the presence of the nucleic acids appeared to cause a slight reduction in the amount of BSA in the alkali fraction (table 4.10b; 58% of the carbon in the fraction) as compared with the amount in table 4.10a (62%). The difference in percentage (4%) was, however, within experimental error.

From these experiments, it was seen that the concentration of BSA detected in the alkali fraction by the Ninhydrin assay corresponded to the percentage detection of carbon in the alkali fractions containing *S. coelicolor* proteins (section 4.4.4: table 4.9). The presence of additional molecules in the biomass may have contributed to further retention of protein within the acid fractions but the extent of retention was unlikely to be significant. Therefore, a possible conclusion was that the alkali fractions did contain the majority of the protein extracted from *S.coelicolor* biomass but that the Ninhydrin and other protein detecting assays did not seem to detect greater than 60% of this protein. Further analyses of *S. coelicolor* biomass therefore used the carbon content of the alkali fraction to determine the concentration of protein present.

## 4.5 Determination of the macromolecular composition of *S. coelicolor* grown in the Bioengineering fermenter

Previous examination of *S. coelicolor* grown in NMM-J in shake flasks had shown that it was difficult to obtain growth and actinorhodin production that was reproducible (section 3.4). It was also difficult to determine a close approximation of the macromolecular composition of the organism (section 4.4.1). Growth of *S. coelicolor* in batch culture conditions in a fermenter had also resulted in large differences between fermentations (section 3.5.1). Nevertheless, because large sample volumes could be removed from the fermenter, which reduced errors caused by pooling cultures, it was possible

that better approximations of macromolecular composition would be obtained using biomass samples grown in the fermenter.

### 4.5.1 Pattern of macromolecular change throughout growth of *S. coelicolor* 1147

During exponential growth of a micro-organism, biosynthesis of macromolecules occurs at a rate similar to the biomass production rate. Therefore, increases in protein, RNA or DNA concentration in a culture may be parallel to that of biomass concentration and could thus be used to measure growth rate (Mandelstam *et al.*, 1982). When cells enter stationary phase, however, the macromolecules undergo separate processes: initiated rounds of DNA replication are completed while RNA and protein degradation occurs to provide precursors for DNA synthesis or newly-synthesized proteins (section 1.5.1).

To determine the pattern of macromolecular change during growth of *S. coelicolor* in NMM-J, three fermentations were performed from which samples were harvested throughout the fermentation periods. The fermentations described correspond to fermentations **a**, **b** and **c** which are depicted in section 3.5.1. The biomass samples from the fermentations were analyzed for macromolecular content using fractionation method 1 (section 2.9.1) and the Burton and orcinol methods of nucleic acid determination (sections 2.10.2 and 2.10.3 respectively). Protein determinations were carried out by carbon analysis (section 2.7.3) of the alkali fractions from the samples. Resulting macromolecular concentrations in the biomass fractions were then expressed as concentrations of the appropriate fermentation cultures and the values for each fermentation were plotted against time of harvesting (figure 4.3). Long time intervals were present between points on the graphs because of the large sample volumes (of up to 1litre) required to allow harvesting of sufficient biomass.

The pattern of macromolecular change in fermentations **a** and **b** followed the patterns expected from the general description of batch growth (section 1.5.1). This description entailed protein, RNA and DNA accumulation during "exponential" growth (it was not clear from growth paterns of *S. coelicolor* that growth was exponential) followed by degradation of RNA and protein during stationary phase. Initial degradation of these macromolecules was presumably to provide precursors for continuing DNA

replication. The concentration patterns exhibited by RNA and protein in fermentation **a** were observed to follow the pattern of biomass concentration during that fermentation (as shown in figure 3.5a). During stationary phase, DNA concentrations in bacterial cultures reach a plateau and this was observed in fermentation **a** at approximately 48 hours of cultivation. Actinorhodin was visible in the culture medium by this time (figure 3.5a) which suggests that the onset of production of actinorhodin occurred before the mycelia entered stationary phase. This supports the observation that the antibiotic was produced during the transient period between exponential growth and stationary phases (section 3.8). A decrease in DNA concentration was observed thereafter in fermentation **a**, with the expected slow rate of degradation possibly associated with cell death.

As stated previously, the patterns of macromolecular change in fermentation **b** were similar to those in fermentation **a**. However, the absolute macromolecule concentrations were several fold less than the concentrations in fermentation **a**: protein was approximately 9-fold less, RNA approximately 3-fold less while the DNA concentration was also almost 3-fold less. These low values correspond to the lower biomass concentrations of fermentation **b** in comparison to fermentation **a**, but differences in macromolecule ratios were also evident. Lysis had occurred relatively early during fermentation **b** (from 35 hours) which may explain the discrepancies in these ratios. However, it is possible, as indicated by the slope of protein concentration, that growth rate was higher during fermentation **b** (until 35 hours) accounting for the higher RNA to protein ratio in this fermentation. Lysis may also explain the observed plateau of RNA concentration, but it is possible that protein degradation may occur sooner and to a greater extent than RNA degradation in *S. coelicolor* biomass. The pattern of DNA concentration in fermentation **b** was comparable to that in fermentation **a** suggesting that viable cells were present throughout the fermentation period although overall lysis had occurred.

The patterns of macromolecular concentrations in fermentation **c** were unlike those in fermentations **a** and **b**. All macromolecules continued to increase throughout the cultivation. Biomass production had reached a maximum value by approximately 50 hours after inoculation, but actinorhodin had been produced simultaneously with biomass from 40 hours. It is possible that a two-stage fermentation had occurred in which a proportion of the mycelium had grown and entered stationary phase while

the remainder continued to grow. The proportion of the population in stationary phase could have produced actinorhodin while biosynthesis of macromolecules continued. The graph of fermentation c may therefore have been the outcome of cells in different physiological states resulting in a heterogeneous fermentation.

### 4.5.2 Macromolecular compositions of biomass grown in the fermenter

The samples used to construct the graphs of macromolecular change in three fermentations (section 4.5.1) were also used in the determination of the macromolecular contents of *S. coelicolor* biomass grown in the fermenter. To check that these samples were suitable for macromolecular determinations, the biomass fractions (resulting from extraction by fractionation method 1; section 2.9.1) were initially compared, by carbon analysis (section 2.7.3), with the fractions from biomass grown in flasks (section 4.4.1). The proportions of carbon in the fractions (table 4.11) were subsequently shown to be consistent with those determined previously (table 4.6): the cold PCA fraction comprised 5% - 12% of the total carbon, the hot PCA fraction contained 12% - 25%, while 50% had been extracted into the alkali fraction and the residue fraction held 12% - 25%.

Macromolecular compositions of the biomass samples from the three fermentations were calculated by expressing the macromolecular content of each fraction as $mg.g^{-1}$ dry weight (dry weight was estimated by carbon analysis assuming 50% carbon, which was based on the carbon content of *E. coli* given by Ingraham *et al.*, 1983; later analysis of *S. coelicolor* biomass (section 4.8) gave 46.7% carbon). Compositions of biomass samples from a further six fermentations were determined (at times only two or three samples were harvested from a fermentation) and the results from two or more samples harvested at similar times were expressed as means (table 4.12). Although the fermenter conditions were assumed to be more constant than flask conditions, the differences observed in growth of *S. coelicolor* 1147 in the fermenter (section 3.5.1) were reflected in the large standard deviations from the means of biomass compositions. These deviations were in a range similar to those from flask grown biomass (section 4.4.1). The mean values of RNA, DNA and protein contents were very similar up to 73 to 82 hours after inoculation showing that the ratios of the macromolecules to biomass were constant during this period. This constancy of ratios usually indicates growth, but in this case may have been a consequence of

macromolecular degradation occurring simultaneously with reductions in biomass concentrations. An approximate macromolecular composition of *S. coelicolor* was, however, obtained from these macromolecular data: 53% protein, 14% RNA and 6% DNA. These values were within the ranges obtained from biomass grown in flasks and were therefore used as macromolecular composition values for determination of monomeric composition.

### 4.5.3 Further analysis of fermenter grown biomass

A further attempt was made to characterize the composition of the macromolecular fractions of biomass grown in the fermenter. Although protein was estimated to account for 100% of the carbon present in the alkali fraction, high percentages of carbon from the cold PCA, hot PCA and residue fractions were unaccounted for (section 4.4.2). The cold PCA fraction undoubtedly contained numerous small molecules (described in section 4.3.1) and the residue fraction consisted largely of peptidoglycan components. However, nucleic acid in the hot PCA fraction generally contributed to 50% of the carbon. Therefore, another molecule which was insoluble in 0.2N PCA had possibly been extracted into this fraction. A potential candidate was teichoic acid which also contains phosphate groups.

Baillie (1968) developed a method for extraction and separation of teichoic acid from the Gram-positive bacterium, *Bacillus subtilis*. The method was based on the nucleic acid extraction protocol of Schmidt and Thannhauser (1945). It involved an initial extraction of small molecules using weak acid, followed by alkaline extraction of nucleic acids, teichoic acid and protein. Subsequent acidification caused precipitation of DNA and protein. For the studies presented here, each step of Baillie's method (Baillie, 1968) was used, but DNA was further extracted into 0.5N PCA at 70°C and protein was extracted into 0.5N NaOH at 37°C (method 2; section 2.9.2).

To compare fractionation methods 1 and 2 (M1 and M2; section 2.9), the distribution of carbon throughout the fractions was determined. A biomass sample was divided, subjected to each method and carbon analysis was carried out with each fraction (table 4.13). Fraction 2 (M2) contains RNA and teichoic acid, and fraction 3 (M2) contains DNA. The combined carbon content of these two fractions was comparable to the carbon content of the hot PCA fraction (M1). Similarly, the amount of carbon in fraction 4 (M2)

and the alkali fraction (M1) were in agreement. However, fractions 1 and 5 (M2), which were supposed to correspond to the cold fraction (M1) and the residue fraction (M1) respectively, were not in accordance. Fraction 1 (M2) contained a higher relative percentage of carbon, with the consequence of a lower relative proportion of carbon in fraction 5 (M2). This may be indicative of more efficient extraction of the small molecules by 0.6N PCA (M2). However, the extraction time for this method (M2) was only 10 minutes whereas in method 1 extraction was by 0.2N PCA overnight. Degradation of nucleic acids and protein, resulting in extraction of the smaller products into fraction 1 (M2), was probably unlikely as the carbon contents of their respective fractions in both methods 1 and 2 were in agreement.

Determination of teichoic acid in the hot PCA fraction (M1) and fraction 2 (M2) was carried out by subtracting nucleic acid phosphate from total phosphate concentration (Baillie, 1968). Measurement of nucleic acid (table 4.14) revealed that ribose and deoxyribose groups were present in both fractions 1 and 2 (M2), possibly indicating inefficient acidification of fraction 2 (M2). Summed amounts of each nucleic acid (estimated by the assays in sections 2.10.2 and 2.10.3) extracted by method 2 were lower than amounts obtained from method 1, but were within 10% error limits. Remaining phosphate contents of fractions 2 and 3 (M2) indicated that a substantial proportion of carbon present in the fractions was derived from an organic phosphate molecule, possibly teichoic acid (table 4.14, TA-P). On comparison with the hot PCA fraction (M1), the remaining phosphate content of fractions 2 and 3 combined (M2) was observed to be lower (after subtraction of nucleic acid phosphate). However, the difference in remaining carbon content (on subtracting nucleic acid carbon) of these combined fractions was shown to be equivalent to the difference in carbon content of fraction 1 (M2; which was greater) and that of the cold PCA fraction (M1). This suggested that more efficient extraction had taken place during the first step of method 2.

Teichoic acid was not quantified directly but, in accordance with Baillie (1968), was assumed to account for the remaining phosphate groups in the appropriate fractions (2 and 3 (M2)). Exact quantitation of carbon derived from teichoic acid was therefore not possible but estimates were calculated using the assumptions that teichoic acid in *S. coelicolor* is composed of either a glycerol backbone or a ribitol backbone. The number of carbons in one unit of teichoic acid would therefore be either 9 or 11 (teichoic acid also

contains an additional group which in this case was assumed to be a sugar group). However, expressing both nucleic acid and teichoic acid in terms of μg of carbon and subtracting the values from the total carbon in the fractions resulted in a substantial amount of remaining carbon. Assuming a glycerol backbone, then 25.8% and 20.5% of the carbon in the hot PCA fraction (M1) and fractions 2 and 3 (M2) respectively were unaccounted for. Assuming a ribitol backbone, 18.1% and 10.7% of the carbon remained unidentified in each method.

It had been shown previously (section 4.4) that the presence of nucleic acid in a standard protein solution caused retention of a small amount of protein in the acid fractions of method 1. In an attempt to identify the source of remaining carbon in fractions 2 and 3 (M2), a similar experiment to that in section 4.4.3, describing extraction of BSA, was carried out using fractionation method 2. Retention of protein within the initial fractions (1 to 3) was observed to occur both in the absence and presence of nucleic acid (table 4.15). The calculated carbon in the nucleic acid fractions of method 2 that was not accounted for by either nucleic acid or possibly teichoic acid may, therefore, have been derived from protein or amino acids.

To check the extent of extraction of carbon into each fraction by method 2, the Baillie method was repeated on triplicate samples. Carbon measurements, however, revealed slight discrepancies in the carbon content of each fraction, especially that of fractions 2 and 3 (table 4.16). In comparison with the sample used previously in this method (table 4.13b), a lower relative amount of carbon in fraction 1 was observed with an almost equivalent relative increase in the combined contents of fractions 2 and 3. This demonstrated that either inefficient or incomplete extraction of carbon into fraction 1 had occurred or residual material had been measured in the first fraction of the previous sample. Phosphate measurements showed the presence of a greater amount of phosphate in fractions 2 and 3 than had been measured in similar fractions of the previous sample. This suggested that incomplete extraction of some small phosphate molecules (organic or inorganic) had occurred. However, the percentages of total carbon present within the first fractions of these triplicate samples were comparable to those in cold PCA fractions (M1) whereas those of fractions 4 were lower then had been previously determined. This indicated possible protein degradation resulting in the extraction of amino acids into fractions 2 and 3, or insufficient removal of residual material from these fractions. Nevertheless, the discovered discrepancies did not render method 2 suitable

for continued analyses and so method 1 was considered more appropriate for these studies.

## 4.6 Determination of the monomeric composition of *S. coelicolor*

Determination of the monomeric composition of *E. coli* given in Neidhardt (1987) was carried out both by analytical measurements and by expression of macromolecular content in terms of monomers. These calculations were based upon, for example, known nucleotide base ratios of RNA and DNA. This approach was applied in the construction of a table of monomeric composition of *S. coelicolor*: known nucleic acid and lipid contents were used and amino acids were quantified directly by HPLC.

### 4.6.1 Estimation of nucleic acid and lipid components of *S. coelicolor*

The ribonucleotide and deoxyribonucleotide contents of *S. coelicolor* were calculated from the RNA and DNA compositions. The GC mole content of *Streptomyces* is very high, 69 - 73% (Pridham and Tresner, 1974; Stanier *et al.*, 1981). This content was therefore used to calculate the amounts of deoxyribonucleotides by assuming approximate proportions of 35% dGMP, 35% dCMP, 15% dAMP and 15% dTMP. This could also have been applied to the proportions of ribonucleotides in mRNA. However, mRNA comprises only 4.0% of total RNA in *E. coli*. The remaining RNA consists of 81.5% rRNA and 14.5% tRNA (Neidhardt, 1987). It was assumed that *Streptomyces* contained similar percentages of these RNA species. Therefore, any bias to base content of total RNA was likely to be influenced by that of rRNA.

The nucleotide sequences of 16S rRNA genes from *S. coelicolor* and *S. lividans* are known (Baylis and Bibb, 1987; Suzuki and Yamada, 1988), as are the sequences of several tRNA genes from *S. coelicolor* (Rokem *et al.*, 1990; Plohl and Gamulin, 1990). Baylis and Bibb (1987) calulated that the 16S rRNA gene of *S. coelicolor* had a GC content of 59%, while Plohl and Gamulin (1990) found that, on average, $tRNA^{gln}$ genes were 57% GC rich whereas $tRNA^{glu}$ genes were 70.3% GC rich. In order to estimate feasible ribonucleotide proportions of *S. coelicolor* total RNA, the percentage content of bases in the sequenced rRNA and tRNA genes were calculated (appendix B). This resulted in an RNA content of 59.2% with 21.8% AMP, 32.9% GMP, 26.3% CMP and 19.2% UMP.

The average fatty acid ($C_{16}$) content of *E. coli* was calculated from total lipid and proportions of $C_{16:0}$, $C_{16:1}$ and $C_{18:0}$ chains (Neidhardt, 1987). Holms (1986) used an average fatty acid content (of $C_{16}$ fatty acids) in his computations of throughputs and fluxes. The lipid content of *S. lividans* is known (U Gräfe, G Reinhardt and D Noack, submitted to Journal of Actinomycetes). *S. lividans* is closely related to *S. coelicolor* and so total lipid and the amounts of lipid species were assumed to be very similar in *S. coelicolor*. Therefore, the data from Gräfe *et al.* (personal communication) were used to calculate the fatty acid content (appendix C). According to these data, *S. lividans* fatty acids are of chain lengths $iC_{14}$ to $iC_{17}$. An average fatty acid chain length of $C_{16}$ was therefore used in the calculation.

*Streptomyces* contain very high proportions of branched-chain fatty acids. For example, 84% of the fatty acids in *S. coelicolor* are branched (Kaneda, 1991). These fatty acids require α-keto acids as precursors which are derived from valine, leucine and isoleucine. Iso-fatty acids are derived from leucine whereas isoleucine is the precursor for anteiso-fatty acids (Kaneda, 1977). The highest percentage of branched chain fatty acids in *S. lividans* was reported to be comprised of iso-fatty acids (Grafe *et al.*, personal communication), therefore a high proportion of leucine was required for their biosynthesis. The amount of isoleucine needed for the anteiso-fatty acids was also calculated (appendix C).

### 4.6.2 Identification and quantitation of amino acids in *S. coelicolor*.

The amino acid composition of *S. coelicolor* 1147 was determined by HPLC (section 2.12). A variety of gradient and isocratic solvent methods were used for good separation of amino acid peaks. These are described in more detail in appendix A which also includes a fuller description of the determination of amino acid concentrations. However, it is important to note that glutamine and asparagine were hydrolyzed to glutamate and aspartate during acid hydrolysis and were therefore determined as these amino acids. Cysteine and tryptophan were not determined by the methods used since acid hydrolysis results in the oxidation of cysteine to cystine (a disulphide) and tryptophan is very unstable (Inglis, 1983).

Four *S. coelicolor* fermentations were carried out to provide eight samples for amino acid analysis. Two samples were harvested from each fermentation: one before visible production of actinorhodin, and one

during actinorhodin production. Samples A, B, C and D were harvested from separate fermentations before antibiotic production, while E, F, G and H were the corresponding samples removed later during the fermentations (table 4.17).

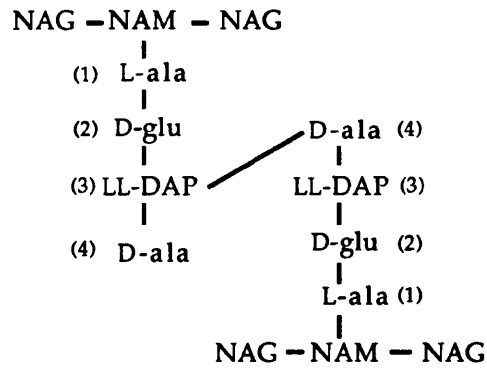
A general pattern of amino acid composition was evident for all samples analyzed. Alanine was the most abundant amino acid, closely followed by glycine; *Streptomyces* have a high proportion of glycine in their cell walls (Pridham and Tresner, 1974). Methionine was the least abundant. Similar amounts of each amino acid were determined in samples C, D, F, G and H. This group contained samples harvested from the same fermentations (C and G, D and H) suggesting little change in amino acid composition of cells after antibiotic production. The content of some amino acids in D and H were slightly higher than in the other samples, especially glutamate, aspartate and histidine. The methionine content was also determined to be much lower in these samples. This possibly reflects the use of different solvent methods for separation of amino acid peaks. As described in appendix A, samples B, C, E, F and G were analyzed by methods which allowed resolution of less common amino acids (appendix A). The resolution of the peaks corresponding to these less common amino acids would have resulted in a decrease in peak size of other amino acids resolved by a different method. For example, a peak corresponding to an α-aminoadipate standard was separated from the glutamate peak by this solvent system. The method by which the amino acids of D and H were determined did not separate these two peaks and so the combined peak was measured as glutamate. Nevertheless, the overall amino acid composition of all the samples were shown to be similar.

Samples A, B and E, however, contained only 50% to 60% of the amino acid contents of the other samples. A and B were harvested from a fermentation in which maximum biomass production was reached at 100 hours after inoculation of the fermenter, *i.e.*, very slow growth. Actinorhodin was not produced at any time. After centrifugation, the harvested samples were in the form of very dense pellets of biomass which were difficult to break up even with sonication. These samples may therefore have contained a high water content resulting in less biomass being analysed. Although the amino acid contents of the samples were low, the relative proportions of individual amino acids were similar, suggesting lack of material was the cause.

The relative proportions of amino acids in each sample were compared by expressing their amounts as percentages of the total amino acid content (table 4.18). As shown in table 4.17, alanine was the most abundant amino acid in every sample, excepting samples A and E. Glycine was present in the highest amounts in these samples, but the amount of threonine was much lower than in other samples. Threonine and glycine peaks co-eluted at times and were difficult to separate. Therefore, determination of these amino acids may have been less accurate in samples A and E.

The percentages of each amino acid were determined to be very close in all the samples analyzed, within 1% to 2% of each other. The means of these percentages were then used to identify positions in the order of decreasing contents. The least abundant amino acids were tyrosine, histidine and methionine. Tyrosine and histidine are also present in low amounts in *E. coli* (table 4.18). However, tryptophan is the least abundant amino acid in *E. coli*, whereas methionine was placed fourteenth among the twenty amino acids. Although alanine and glycine are also present in the highest amounts in the enterobacterium, their percentages are lower than those in *S. coelicolor* by 3% to 5%. Further analysis of the streptomycete amino acid composition may lower this difference, but it is unlikely that the undetermined amino acids (cysteine and tryptophan) would account for the difference. Proportions of the majority of amino acids agree in the two bacteria, therefore, cysteine and tryptophan may comprise approximately 2% and 1% of the total amino acids respectively (these values were used in calculation of the throughputs in *S. coelicolor*; section 5.3.1). Combining the *E. coli* percentage contents of glutamate and asparate with their amidated forms (glutamine and asparagine) resulted in higher values than those obtained from *S. coelicolor*. It may be that the samples had lower amounts of these amino acids or that complete detection of both types of amino acid had not occurred. The glutamate and aspartate values were used, nevertheless, in the determination of throughputs (secton 5.3.1).

Determination of the amino acid compostion had been carried out using whole biomass samples. Fractionation of these samples (section 2.9.1) resulted in a final fraction presumed to contain cell wall-associated components, *e.g.*, peptidoglycan (section 4.3.1). Peptidoglycan is a polymer generally comprised of N-acetylglucosamine (NAG), N-acetylmuramic acid (NAM), L(D)-alanine (L(D)-ala), D-glutamic acid (D-glu) and *meso*-diaminopimelic acid (*meso*-DAP). *Streptomyces* peptidoglycan, however, contains LL-DAP in the structure:




(Goodfellow and Cross, 1984; Minnikin and O'Donnell, 1984: the residue position numbers are given in brackets), and an interpeptide bridge of 1 to 6 amino acids long exists between positions 3 and 4 (Minnikin and O'Donnell, 1984; Stanier *et al.*, 1981). Streptomycete cell walls are also reported to contain a high quantity of glycine (Minnikin and Cross, 1984, Pridham and Tresner, 1974). Therefore, to verify the content of the residue fractions and to quantify peptidoglycan from *S. coelicolor*, amino acid analysis was performed on the residue fractions of samples A, B, C, D and F (Table 4.19).

Several of the amino acids present in whole biomass samples of *S. coelicolor* were detected in the residue fractions. α-Aminoadipate and N-acetylglucosamine were also present and were quantified with the common amino acids. According to Neidhardt (1987), *E. coli* peptidoglycan consists of N-acetylglucosamine, alanine and glutamate in the ratio 1:2:1. The ratios of these amino acids in the *S. coelicolor* residue samples were calculated with respect to N-acetylglucosamine:

| sample | NAG | ala | glu |
|---|---|---|---|
| A | 1.0 | 2.4 | 2.0 |
| B | 1.0 | 2.1 | 1.0 |
| C | 1.0 | 2.2 | 1.8 |
| E | 1.0 | 1.7 | 0.8 |
| F | 1.0 | 1.9 | 0.9 |

The low value of N-acetylglucosamine to glutamate in samples A and C may have been due to the detection of residual amounts of glutamate derived from soluble protein. Residual amounts of other amino acids may also have been present. However, it is possible that some of the amino acids detected in the residue fractions were components of an interpeptide bridge in the peptidoglycan molecule. The average amount of N-

acetylglucosamine present in the residue fractions was 51.4μmole.$g^{-1}$ dry weight. Peptidoglycan has a molecular weight of 904 giving a weight of 46.5mg.$g^{-1}$ and, therefore, 4.6% of *S. coelicolor* dry weight. *E. coli* contains 2.5% peptidoglycan (Neidhardt, 1987).The presence of a relatively high quantity of glycine also indicated that the residue fractions contained additional cell wall material.

## 4.7 Compiled macromolecular and monomeric compositions of *S. coelicolor*.

The analyses carried out using *S. coelicolor* biomass and described in the previous sections were used to compile a macromolecular composition of this organism. From spectrophotometric determinations and carbon analyses of biomass grown in flasks and the fermenter (sections 4.1, 4.4.2, 4.5.2) an approximate macromolecular composition was considered to be 53% protein, 14% RNA, 6% DNA, 5.5% lipid and 4.6% peptidoglycan. This demonstrated that 83.1% of *S. coelicolor* biomass had been accounted for. The remaining proportion of biomass was proposed to consist of teichoic acid and additional unmeasured components. Teichoic acid measurements were not included in the macromolecular composition because the determined measurements of phosphate, presumably derived from teichoic acid, were calculated to be 9.0% of biomass. This appeared unfeasible since teichoic acid supposedly comprises 20% of streptomycete cell walls (Naumova *et al.*, 1978; cited by Locci and Sharples, 1984) whereas up to 80% of the streptomycete cell wall dry weight is peptidoglycan (Minnikin and O'Donnell, 1984; Stanier *et al.*, 1981).

Determination of the monomeric composition of *S. coelicolor*, both by calculation and analysis, has been described in section 4.6. The data of samples C, D, A and B (section 4.6) were compiled in a form similar to that of *E. coli* given in section 4.1 (tables 4.20a, b, c and d respectively; note, *S. coelicolor* data are expressed in mmol.$g^{-1}$ dry weight). The values in tables 4.20a and b are comparable to those of *E. coli*, especially the amino acid values. However, since the analyses were preliminary, additional compositional analyses should be carried out on *S. coelicolor* to allow better comparison with the *E. coli* composition. Tables 4.20 c and d contain lower amino acid contents (by approximately two-fold) and lower nucleotide contents. These samples were harvested from *S. coelicolor* fermentations that did not reach their maximal concentrations of biomass until almost 100

hours after inoculation (other fermentations reached this stage at 40 to 60 hours). It is possible, therefore, that samples A and B were harvested prior to "exponential" growth phase as the metabolism of the mycelia was adjusting for growth in NMM-J, *i.e.*, during an extended lag phase. Nevertheless, the monomeric composition data of samples C and D were sufficient for determining throughputs and fluxes by Holms' method (Holms, 1986; see chapter 5).

## 4.8 Elemental composition of *S. coelicolor* biomass.

The macromolecular and monomeric compositions of samples of *S. coelicolor* biomass have been given in the preceding section. Further compositional analysis involves the elements which allow mass balance equations to be formulated. These equations represent the stoichiometry of the thousands of chemical reactions required to produce bacterial biomass (section 1.10). Elemental analysis of *S. coelicolor* was performed on samples removed from a chemostat culture (in Jena, Germany; section 3.7). *S. coelicolor* had been grown at a growth rate of $0.04h^{-1}$ in a phosphate-limited medium at the time of harvesting. Although lysis had occurred in these samples (section 3.7.6), it was possible to carry out analysis for the elements carbon (C), hydrogen (H) and nitrogen (N). Insufficient material was available for phosphorous and sulphur determinations. Means of percentage contents of the elements from five replicate samples (analyzed in duplicate) were combined to give an average composition of *S. coelicolor*. The oxygen (O) content was calculated by sutraction from 100%. The empirical formula of the biomass was computed by expressing each element as number of gram atoms (by dividing the percentage value of the elements by their specific atomic masses). The values were then normalised with respect to a carbon unit of 1.0.

| | | | | |
|---|---|---|---|---|
| Elemental composition | C | 46.68 +/- 0.48 | | |
| | H | 7.23 +/- 0.16 | | |
| | N | 8.02 +/- 0.11 | | |
| | O | 38.07 (by subtraction). | | |
| Number of gram atoms | C | 3.89 | N | 0.57 |
| | H | 7.23 | O | 2.38 |

| | |
|---|---|
| Empirical formula | $CH_{1.86}O_{0.61}N_{0.15}$ |
| Molecular mass | 25.72g. |

In comparison to the elemental compostion of *E. coli* (section 4.1; Ingraham *et al.*, 1983), the composition of *S. coelicolor* was determined to be very similar in respect of carbon and hydrogen content (a carbon content of 50% had been assumed for wet weight estimations). However, *E. coli* contains 20% O and 14% N, with an empirical formula of $CH_{1.9}O_{0.3}N_{0.24}$. According to Roels (1980), "most micro-organisms" have an empirical formula of $CH_{1.65}O_{0.52}N_{0.2}$. The higher oxygen content of *S. coelicolor* was also observed in elemental compositions of two other streptomycete species, *S. thermonitrificans* ($CH_{1.85}O_{0.61}N_{0.22}$; Burke, 1991) and *S. cattleya* ($CH_{1.6}O_{0.58}N_{0.17}$; Bushell and Fryday, 1983). *Streptomyces* are obligate aerobes and it may be that the oxidation state (reflected by the oxygen content) in these organisms is higher than in, *e.g.*, facultative anaerobes.

Fermentation mass balance equations were formed using the formula for the biomass of *S. thermonitrificans* (Burke, 1991) and *S. cattleya* (Bushell and Fryday, 1983). However, the *S. coelicolor* biomass samples had been harvested when the culture was growing at a very low growth rate, and the biomass yield had been determined to be very low in *S. coelicolor* batch fermentations (section 3.5.1). Therefore, attempts at producing a mass balance equation resulted in figures incomparable with those of the other streptomycete fermentations.

## 4.9 Discussion

The composition of *E. coli*, the organism used by Holms (1986) in his example of the computation of fluxes, was based on that given in Morowitz (1968). An alternative composition of *E. coli*, given by Neidhardt (1987), was a compilation of the work of numerous groups over many years. This composition was used to compare that of *S. coelicolor* biomass samples.

The *S. coelicolor* data given in this chapter were from initial analytical studies performed during this singular study. Several components of *S. coelicolor* were not measured and some measurements were omitted from the final monomeric composition tables. Nevertheless, the available data

were sufficient to compute flux diagrams giving a general pattern of the activity of the central metabolic pathways of *S. coelicolor* 1147 (chapter 5).

Initial analyses were concerned with the macromolecular composition of *S. coelicolor* 1147 grown in flasks. The cultures had been grown in NMM-J (section 2.5.1) because the inclusion of junlon in the medium had caused interference in both the orcinol and anthrone assays (for RNA and carbohydrate respectively). Macromolecular determinations were carried out on several biomass samples harvested at similar times. All samples had been cultivated under similar conditions and it was therefore expected that macromolecular compositions would be comparable between samples harvested at similar times. This was based on the observations of Schaechter *et al.* (1958; section 1.5.4) that bacterial species grown in medium of the same composition grew at the same growth rate and had the same chemical composition. However, analysis of *S. coeliciolor* samples resulted in values of great variability. This reflected the observed diferences in growth (and antibiotic production) of cultures cultivated in flasks (section 3.4). Pooling of cultures did not minimize the variabilities in macromolecular composition. Large standard deviations were also obtained when samples grown in flasks were compared with respect to the phase of antibiotic production. It was possible that *S. coelicolor* grown under minimal conditions was very sensitive to minute, perhaps undetectable, differences in the culture conditions. Nevertheless, a rough macromolecular composition of *S. coelicolor* was proposed from these evaluations: 8-15% RNA, 4-6% DNA and 12-21% protein.

The range of values for RNA were lower than the RNA content of *E. coli*, possibly reflecting a slower rate of growth. DNA values were comparable to that of *E. coli*. However, protein estimations (by the Bradford assay) were much lower than expected. Carbon analysis of the alkali fractions, into which protein was extracted, showed that the fractions (presumed to contain only protein) comprised 50% of the total carbon of the biomass. Expression of the measured protein as carbon showed that this estimation accounted for less than 50% of the carbon in the alkali fraction. Investigations into reasons for this included the use of alternative methods of protein determination but protein estimations by all of these methods were low. To account for any protein degradation occurring during alkali extraction, amino groups present in the fraction were assayed. However, this still did not account for 100% of the carbon-containing molecules. Fractionation of a BSA standard revealed that the Ninhydrin assay did not detect all the BSA

present in the alkali fraction. The use of BSA as a standard was therefore not suitable for protein measurements extracted by 0.5N NaOH (as in the alkali fraction). Carbon analysis was possibly more accurate than using other protein standards, therefore, the amount of carbon present in the alkali fraction was used as a measure of the amount of protein extracted from *S. coelicolor* biomass.

Subsequent determinations of the macromolecular composition of *S. coelicolor* were performed on samples harvested from fermentations. These were carried out in the Bioengineering fermenter. It was thought that the use of the fermenter would reduce the differences in composition of samples harvested at the same time. However, possible sensitivity of *S. coelicolor* to minute changes in environment was also exhibited in the fermenter and different growth patterns were observed between fermentations. This was shown by studying the patterns of macromolecular change in fermenter cultures. Therefore, macromolecular compositions of samples also varied. Nevertheless, an approximate macromolecular composition was proposed: 53% protein, 14% RNA and 6% DNA. These values agreed with previous determinations of flask grown biomass and *S. coelicolor* 209 biomass (section 3.3.2).

An attempt was made to characterize further an additional molecule present in *S. coelicolor* biomass, *i.e.*, teichoic acid. This involved the use of an alternative method of fractionation. Teichoic acid was assumed to be extracted into the same fraction as RNA. However, phosphate and carbon measurements of this fraction were difficult to correlate and resulted in probable overestimation of teichoic acid. It was possible, however, that other molecules such as amino acids had been extracted into the RNA and teichoic acid fraction which would account for some of the excess carbon. Amino groups had been extracted into this fraction during fractionation of a BSA standard.

Macromolecular compositions were used to calculate the amounts of some monomeric components present in *S. coelicolor* for the compilation of monomeric composition data. This included the nucleic acid contents and their respective nucleotides. The GC content of *Streptomyces* DNA is known as approximately 70% and it was possible to calculate the nucleotide content of *Streptomyces* RNA. Also included was the fatty acid composition of *S. coelicolor*. However, the lipid content was taken from analysis of *S. lividans*. *S. coelicolor* and *S. lividans* are very closely related (Kieser *et al.*,

1992) and *S. lividans* produces actinorhodin and undecylprodigiosin under certain conditions (*e.g.*, in the presence of *afsR;* section 1.7.2). It was assumed therefore, that the lipid composition of both species would be similar.

The amino acid composition of the proteins of *S. coelicolor* was measured directly by HPLC. The compositions obtained were comparable to the amino acid content of *E. coli,* with alanine as the most abundant amino acid followed by glycine. However, these amino acids comprised a higher proportion of the total amino acid content in *S. coelicolor.* Methionine was the least abundant. Other amino acids in *S. coelicolor* were different in their order of abundance in comparison to those in *E. coli.* It is possible that the high GC content of *Streptomyces* influences the requirement for amino acids. Some amino acids with AT rich codons may be replaced by those with less AT rich codons (see chapter 6 for further discussion).

Amino acid analysis also confirmed the presence of cell wall material in the residue fractions of fractionated biomass samples. This was based on detection of N-acetylglucosamine, a component of peptidoglycan. The ratios of N-acetylglucosamine to alanine and glutamate in these fractions were similar to those in *E. coli* peptidoglycan. Glycine, a major amino acid in *Streptomyces* cell walls was also present at high concentration.

Tables of monomeric compositions of four *S. coelicolor* biomass samples harvested prior to actinorhodin production were constructed using the data obtained. The values of the first two samples (C and D) were very similar whereas those of A and B were lower. This may reflect the amount of biomass analyzed. Dry weight estimations and carbon analysis had shown that samples harvested early in fermentations contained less dry weight biomass per gram wet weight (and therefore more water) than samples harvested later (section 3.5.1). An alteration in mycelial morphology at the onset of antibiotic production had been proposed to account for this (section 3.5.1). Biomass pellets harvested early were also more difficult to resuspend for the purpose of analysis. It was possible, therefore, that the amounts of biomass in samples A and B were not equivalent to that in samples C and D. It is also possible that the samples were harvested during a long lag phase and the macromolecules had not reached the maximum proportions for $\mu_{max}$. Comparison of the deoxyribonucleotide content of the four samples did reveal similarities (the lower ribonucleotide values of A and B may reflect lower growth rate). This suggests that analysis of a smaller amount of

material was the reason for the lower amino acid contents of samples A and B.

The monomeric compositions of C and D were comparable to *E. coli* with the slight differences expected between Gram-positive and Gram-negative bacteria. The main differences were in deoxyribonucleotide content and peptidoglycan content. However, comparison of the nucleic acid content of *S. coelicolor* with that of *E. coli* grown at a slower growth rate revealed similarities, and since Gram-positive species contain more peptidoglycan than Gram-negative species, the amount of peptidoglycan was feasible. The results obtained from these initial analyses therefore provide a reasonable foundation upon which further determinations of the chemical composition of *Streptomyces* (*S. coelicolor* in particular) can be based.

Compositional determinations of an organism would not be complete without expressing the biomass in terms of its elements. Analysis of the elemental composition of *S. coelicolor* resulted in an empirical formula of $CH_{1.86}O_{0.61}N_{0.15}$. This was very similar to the elemental compositions of two other streptomycete species, *S. cattleya* (Bushell and Fryday, 1983) and *S. thermonitrificans* (Burke, 1991). Bushell and Fryday (1983) had compared the elemental composition of *S. cattleya* with those reported for other organisms and observed that *S. cattleya* biomass contained a higher proportion of oxygen and was therefore in a more oxidized state (section 4.8). Oxygen was also present at a high proportion in *S. coelicolor* biomass. Most of the organisms compared with *S. cattleya* and also with *S. thermonitrificans* (Burke, 1991) were facultative anaerobes. If any poorly aerated areas are present in cultures, these micro-organisms have the ability to survive using either alternative electron acceptors (*e.g.*, nitrate) or fermentative respiration. For example, in flask cultures *Saccharomyces cerevisiae* may exhibit the Crabtree effect, *i.e.*, alcoholic fermentation occurs in aerobic conditions in the presence of excess glucose (van Dijken and Scheffers, 1986), in addition to normal fermentation because of a limiting factor in the cultures. Therefore, the empircal formulae for these facultative anaerobes may be reflective of growth in both aerobic and anaerobic conditions. *Streptomyces*, however, are obligate aerobes. Growth of streptomycete mycelia cannot occur in small unaerated areas in cultures. It is possible that because they require oxygen to grow and do not undergo anaerobic or fermentative respiration, *Streptomyces* retain a high proportion of oxygen in their biomass. An alternative hypothesis is that

because *Streptomyces* biomass is more oxidised, they require a high oxygen content for growth.

The biomass used to determine the elemental compositions of *S. cattleya* and *S. thermonitrificans* (Bushell and Fryday, 1983; Burke, 1991) was taken from exponentially-growing batch cultures. However, the biomass used for analysis of *S. coelicolor* had been acquired from the chemostat (in Jena, Germany) in which *S. coelicolor* was growing at a growth rate of $0.04h^{-1}$. The limiting nutrient was phosphate. In batch cultivations, *S. coelicolor* had reached a $\mu_{max}$ of $0.12h^{-1}$. In a study of the growth of *Klebsiella aerogenes* at different growth rates in continuous culture, the elemental composition was observed to be similar at different growth rates (Mulder *et al.*, 1988). It is possible, therefore, that in batch culture *S. coelicolor* has an elemental compostion similar to that at a growth rate of $0.04h^{-1}$.

In conclusion, analysis of *S. coelicolor* biomass resulted in compositional data at the macromolecular, monomeric and elemental levels. The values obtained, however, are not definitive and further work is required to obtain information comparable to that reported for *E. coli*. Nevertheless, the monomeric compositions of biomass samples were sufficient to calculate throughputs and, therefore, fluxes to biomass through the central metabolic pathways of *S. coelicolor* (chapter 5).

**Table 4.1. Monomeric composition of *Escherichia coli* (from Neidhardt, 1987).**

| Residues | Amt (μmol/g of dried cells) | Residues | Amt (μmol/g of dried cells) |
|---|---|---|---|
| Protein amino acids | | Lipid components | |
| Alanine | 488 | Glycerol | 161 |
| Arginine | 281 | Ethanolamine | 97 |
| Asparagine | 229 | $C_{16:0}$ fatty acid (43%) | |
| Aspartate | 229 | $C_{16:1}$ fatty acid (33%) | |
| Cysteine | 87 | $C_{18:1}$ fatty acid (24%) | |
| Glutamate | 250 | Average fatty acid | 258 |
| Glutamine | 250 | | |
| Glycine | 582 | LPS components | |
| Histidine | 90 | Glucose | 16.8 |
| Isoleucine | 276 | Glucosamine | 16.8 |
| Leucine | 428 | Ethanolamine | 25.2 |
| Lysine | 326 | Rhamnose | 8.4 |
| Methionine | 146 | Heptose | 25.2 |
| Phenylalanine | 176 | KDO | 25.2 |
| Proline | 210 | Hydroxymyristic acid | 33.6 |
| Serine | 205 | Fatty acid ($C_{14:0}$) | 16.8 |
| Threonine | 241 | | |
| Tryptophan | 54 | | |
| Tyrosine | 131 | Peptidoglycan components | |
| Valine | 402 | *N*-Acetylglucosamine | 27.6 |
| | | *N*-Acetylmuramic acid | 27.6 |
| RNA nucleotides | | Alanine | 55.2 |
| AMP | 165 | Diaminopimelate | 27.6 |
| GMP | 203 | Glutamate | 27.6 |
| CMP | 126 | | |
| UMP | 136 | | |
| | | Glycogen components (glucose) | 154 |
| DNA nucleotides | | | |
| dAMP | 24.6 | | |
| dGMP | 25.4 | Polyamines | |
| dCMP | 25.4 | Putrescine | 34.1 |
| dTMP | 24.6 | Spermidine | 7.0 |

LPS, lipopolysaccharide.

**Table 4.2 Interference by junlon in the Orcinol and Anthrone assays.**

**a** RNA Determination - Orcinol method

| sample | $A_{665}$ | RNA ($mg.ml^{-1}$) |
|---|---|---|
| X | 1.101 +/- 0.045 | 0.362 |
| Y | 0.062 +/- 0.021 | nd |

**b** CHO Determination - Anthrone method

| sample | $A_{620}$ | CHO ($mg.ml^{-1}$) |
|---|---|---|
| X | 0.493 +/- 0.024 | 0.544 |
| Y | 0.689 +/- 0.024 | nd |

*S. coelicolor* 209 biomass grown in HMM was harvested after 72 hours; the pellet (4g, wet weight) was subjected to a fractionation method at a final concentration of $50mg.ml^{-1}$, described in section 2.9.1. A pellet of equivalent weight of junlon, acquired by the centifugation of HMM devoid of mycelia, was fractionated in parallel. The cold and hot PCA fractions were assayed by the orcinol (**a**) and anthrone (**b**) methods respectively (section 2.10.3 and section 2.10.1). A, absorbance measurements at 665nm; X, biomass plus junlon; Y, junlon alone; nd, not determinable.

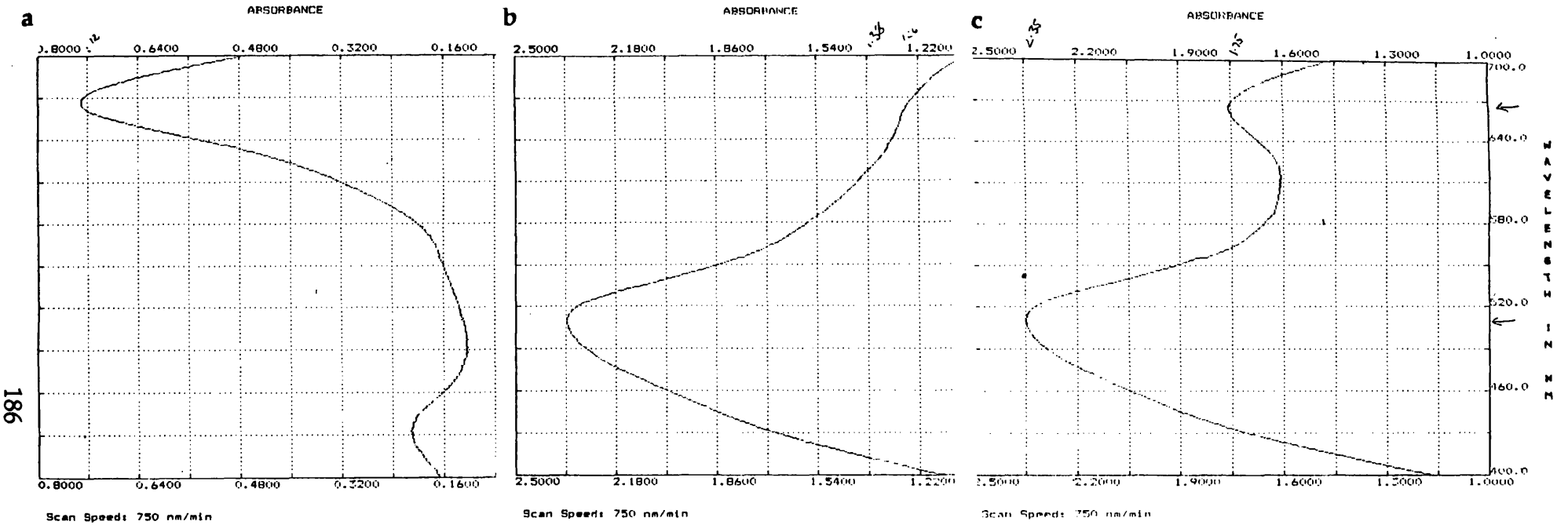



**Figure 4.1. Spectrophotometric scan of products of the orcinol reaction.**
Three substrates for the orcinol reaction were assayed as described in section 2.10.3. The absorbances of the products were then scanned between 400nm and 700nm. All products were measured against an appropriate blank.
**a** 150µg RNA standard. **b** 1.5ml junlon (10% (w/v) wet junlon obtained by centrifugation of HMM). **c** 150µg RNA plus 1.5ml of 10% (w/v) junlon.

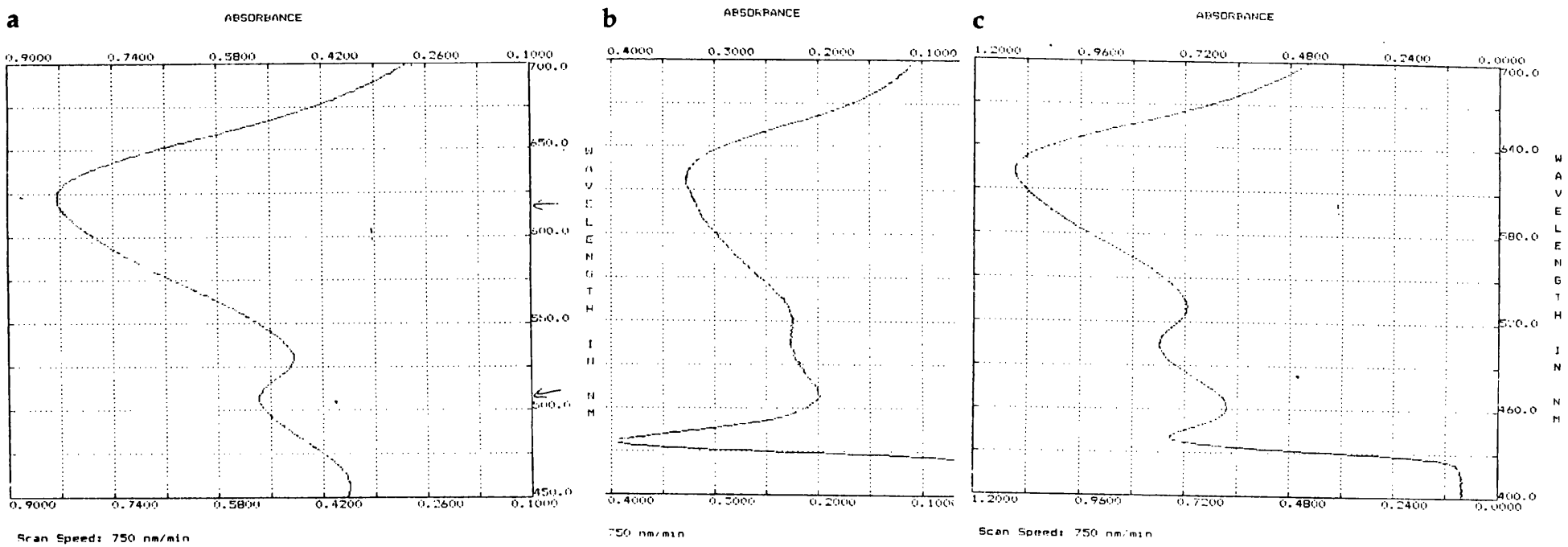



**Figure 4.2. Spectrophotometric scan of products of the anthrone reaction.**
Substrates for the anthrone reaction were assayed by the anthrone method (section 2.10.1). The absorbances of the products were then measured by scanning in a wavelength range of 400nm to 700nm. All products were measured against an appropriate blank.
**a** 100µg glucose standard. **b** 1.5ml junlon (10% (w/v) wet junlon obtained by centrifugation of HMM). **c** 100µg glucose in 1.5ml 10% (w/v) junlon.

**Table 4.3 Determination of the reproducibility of the fractionation and spectrophotometric methods used for the macromolecular estimation of *S. coelicolor* biomass.**

| Sample | CHO | RNA | DNA | PROTEIN |
|---|---|---|---|---|
| 1a | 1.54 | 7.65 | 2.50 | 4.75 |
| 1b | 1.58 | 7.95 | 2.79 | 4.51 |

| Sample | CHO | RNA | DNA | PROTEIN |
|---|---|---|---|---|
| 2a | nd | 6.75 | 2.73 | nd |
| 2b | nd | 6.60 | 2.70 | nd |

| Sample | CHO | RNA | DNA | PROTEIN |
|---|---|---|---|---|
| 3a | nd | 6.64 | 3.72 | nd |
| 3b | nd | 6.68 | 3.84 | nd |

Biomass obtained from three cultures grown from *S. coelicolor* 1147 spores was harvested at 72 hours after inoculation. Each washed pellet was divided into two samples of equal weight which were fractionated (section 2.9.1) at a concentration of 100mg (wet weight).$ml^{-1}$. Resulting fractions were analyzed in duplicate for macromolecular content by the appropriate assay (section 2.10). All figures are expressed as $mg.g^{-1}$ wet weight. nd, not determined.

**Table 4.4. Quantitation of macromolecular concentrations in flask cultures of *S. coelicolor* 1147.**

| group | hours | CHO | RNA | DNA | PROTEIN |
|---|---|---|---|---|---|
| **A** (2)[x] | 48.5 | 7.11 +/- 4.8 | 117.25 +/- 28 | 56.21 +/- 18.3 | 186.37 +/- 3.0 |
| **B** (3) | 56 | 16.34 +/- 6.7 | 71.34 +/- 35.2 | 58.11 +/- 11.6 | 182.22 +/- 15.4 |
| **C** (4) | 64 | 21.44 +/- 8.2 | 81.81 +/- 14.9 | 36.31 +/- 4.7 | 212.97 +/- 58.1 |
| **D** (7) | 73 | 24.05 +/- 6.1 | 110.77 +/- 25.9 | 51.07 +/- 11.5 | 124.35 +/- 72.1 |
| **E** (2) | 85 | 31.52 +/- 10.6 | 156.54 +/- 16.3 | 52.2 +/- 2.5 | 87.87 +/- 1.6 |
| **F** (3) | 96 | 6.19 +/- 2.8 | 57.47 +/- 40.6 | 16.33 +/- 9.6 | 83.23 +/- 60.8 |
| **G** (2) | 120 | 8.94 +/- 4.8 | 94.36 +/- 44.6 | 37.78 +/- 33.8 | 196.8 +/- 112.5 |

53 flasks containing 50ml of NMM-J were inoculated with fresh *S. coelicolor* 1147 spores to give approximately $1x10^7$spores.ml$^{-1}$. All cultures were cultivated under the same conditions (30°C, 250rpm; section 2.6.1). 23 biomass samples were harvested from individual cultures or pooled cultures (2, 3, 4 or 5 flask cultures) at the indicated times (hours). Each sample was then subjected to fractionation method 1 (section 2.9.1) at a concentration of 100mg.ml$^{-1}$ wet weight. Macromolecular components were determined by their appropriate assay (section 2.10). All values are expressed as mg.g$^{-1}$ dry weight (dry weight was based on the carbon content of the biomass) and are denoted as means plus/minus their standard deviations. The means were obtained from biomass samples harvested at the same time (irrespective of antibiotic production) which are classed in groups. The numbers of cultures (x) used in each group are given in brackets after each group letter (first column). CHO, carbohydrate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

**Table 4.5. Quantitation of macromolecular composition of *S. coelicolor*. Comparison of compositions at different physiological states.**

| colour[y] | CHO | RNA | DNA | PROTEIN |
|---|---|---|---|---|
| white | 23.86 +/- 1.5 | 94.82 +/- 7.7 | 65.40 +/- 8.5 | 137.81 +/- 73.1 |
| red | 14.22 +/- 13.0 | 94.20 +/- 32.3 | 48.42 +/- 16.1 | 177.81 +/- 10.8 |
| red/blue | 15.19 +/- 8.3 | 77.14 +/- 27.4 | 34.17 +/- 15.6 | 155.22 +/- 78.5 |
| blue | 22.80 +/- 10.7 | 122.19 +/- 39.4 | 45.1 +/- 18.7 | 174.5 +/- 146.0 |

Biomass compositions of the 23 flask cultures described in table 4.4 were re-grouped in terms of the extent of antibiotic production, irrespective of time of harvesting. Means and standard deviations were recalculated. All values are expressed as $mg.g^{-1}$ wet weight. The colour (y) of each group of cultures was representative of the physiological state of the biomass: white, no antibiotics produced therefore the mycelia only underwent primary metabolism; red, visible production of undecylprodigiosin; blue, visible production of actinorhodin; red/blue, simultaneous production of both antibiotics.

**Table 4.6. Carbon content of fractions expressed as a percentage of biomass carbon.**

| group | COLD (%) | HOT (%) | ALKALI (%) | RESIDUE (%) |
|---|---|---|---|---|
| **A** (1) | 5.4 | 23.8 | 56.4 | 14.5 |
| **B** (3) | 5.1 +/- 0.8 | 8.7 +/- 2.2 | 52.7 +/- 5.5 | 33.5 +/- 3.7 |
| **C** (4) | 10.3 +/- 2.7 | 16.9 +/- 4.5 | 53.8 +/- 4.5 | 19.1 +/- 2.5 |
| **D** (6) | 9.6 +/- 3.2 | 18.6 +/- 2.1 | 50.1 +/- 4.0 | 21.6 +/- 4.9 |
| **E** (2) | 8.4 | 22.0 | 48.9 | 20.8 |
| **F** (1) | 6.0 | 17.8 | 53.9 | 22.3 |
| **G** (1) | 5.6 | 19.1 | 54.8 | 20.5 |

Fractionation of the groups of *S. coelicolor* biomass samples described in table 4.4, from 23 biomass samples, resulted in four supernatants: COLD, cold PCA; HOT, hot PCA; ALKALI and RESIDUE (section 2.9.1; 4.3.1). Each fraction was subjected to carbon analysis using the TOCA (section 2.7.3), the results of which were expressed as ppm, *i.e.*, $\mu g.ml^{-1}$. The carbon content of total biomass (at a concentration of 100mg wet $weight.ml^{-1}$) was also determined. Carbon contents of each fraction were subsequently calculated as percentage of total biomass carbon.

**Table 4.7. Comparison of carbon content of fractions determined from macromolecule estimations and direct carbon measurements.**

| group | CHO | COLD | % | RNA | DNA | HOT | % | PROT | ALK | % | PROT | RES | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 5.2 | 96.8 | 5 | 162.4 | 86.4 | 428.4 | 58 | 322.5 | 1014 | 32 | 52.5 | 260.2 | 20 |
| B | 29.7 | 118.7 | 25 | 149.8 | 97 | 203.4 | 121 | 428.3 | 1253.3 | 34 | 93.8 | 796.2 | 12 |
| C | 41.7 | 262 | 16 | 114.7 | 55.6 | 331.7 | 51 | 405 | 1265.6 | 32 | 29.6 | 406.6 | 7 |
| D | 63.8 | 207.2 | 31 | 169.5 | 78.8 | 428.4 | 59 | 183.8 | 1067.1 | 17 | 74.4 | 522.5 | 14 |
| E | 60 | 201 | 30 | 253.8 | 89.3 | 527 | 65 | 211.2 | 1171.8 | 18 | 92.5 | 496.4 | 19 |
| F | 28.6 | 132.2 | 22 | 184 | 60.4 | 391 | 62 | 505 | 1182.9 | 43 | 106 | 485.5 | 22 |
| G | 28.6 | 162.4 | 18 | 246.7 | 127.4 | 558 | 67 | 800 | 1596.4 | 50 | 117.5 | 597.9 | 20 |

23 biomass samples of *S. coelicolor* 1147, described in table 4.4, were subjected to the fractionation method described in section 2.9.1. Macromolecule concentrations in each resulting fraction (cold PCA, hot PCA, alkali and residue) were determined by the assays described in section 2.10 (protein was measured by the Bradford assay, section 2.10.4.1) and expressed as parts per million (ppm = $\mu g.ml^{-1}$) of carbon (section 4.4.2) in the columns headed CHO (carbohydrate), RNA, DNA and PROT (protein). These values were then calculated as percentages (%) of the total carbon in the appropriate fraction (COLD, HOT, ALK and RES) as measured by carbon analysis (section 2.7.3). All values (excluding percentages) are in ppm of carbon.

**Table 4.8. Protein estimation by the Lowry, Bradford and BCA methods of determination.**

**a** Protein determination by the Lowry method using standards dissolved in 0.5N NaOH.

| dry weight ($g.l^{-1}$) | protein ($mg.l^{-1}$) | protein ($mg.g^{-1}$ d w.) |
|---|---|---|
| 0.023 | 3.0 | 130.43 |
| 0.026 | 5.75 | 216.98 |
| 0.023 | 6.02 | 261.96 |

**b** Protein determination by the Bradford and BCA methods.

| | Bradford method | | BCA method | |
|---|---|---|---|---|
| dry weight ($g.l^{-1}$) | protein ($mg.l^{-1}$) | protein ($mg.g^{-1}$ d w.) | protein ($mg.l^{-1}$) | protein ($mg.g^{-1}$ d w.) |
| 0.060 | 11.4 | 190.95 | 10.44 | 174.87 |
| 0.028 | 5.08 | 180.78 | 5.57 | 198.22 |
| 0.046 | 12.25 | 266.30 | 8.30 | 180.43 |

Three *S. coelicolor* 1147 biomass samples grown in shake flasks were subjected to fractionation method 1 (section 2.9.1) and the alkali fractions were assayed in triplicate for soluble protein (**a**) by the Lowry method (section 2.10.4.2). A further three biomass samples were fractionated and soluble protein in their alkali fractions was measured by (**b**) the Bradford (section 2.10.4.1) and BCA (section 2.10.4.3) methods. The values were measured as $\mu g.ml^{-1}$ of the fraction, obtained from a known wet weight of biomass, and calculated as per litre of culture. Subsequent expression of the protein values as $mg.g^{-1}$ of dry weight (dw; as determined by carbon analysis, section 2.7.3) allowed the protein content of the biomass to be determined.

**Table 4.9. Determination of protein content of alkali fractions as estimated by the Ninhydrin assay and carbon analysis.**

| | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| | AKALI | amino acid (digested) | | amino acid (undigested) | (II-IV) | |
| group | carbon (ppm) | carbon (ppm) | % | carbon (ppm) | carbon (ppm) | % |
| A (1)[x] | 1014 | 644.4 | 63.6 | 217.2 | 427.2 | 42.1 |
| B (3) | 1253.3 +/- 281.2 | 744.6 +/- 108.9 | 59.4 | 292.5 +/- 51.6 | 452.1 | 36.1 |
| C (3) | 1265.6 +/- 196.9 | 640.2 +/- 10.2 | 50.6 | 307.2 +/- 112 | 287.2 | 22.7 |
| D (3) | 1363.4 +/- 125.9 | 486.6 +/- 195.4 | 35.7 | 424.8 +/- 8.7 | 179.4 | 13.2 |
| E (2) | 1171.8 | 688.5 +/- 14 | 58.8 | 431.4 | 263.7 | 22.5 |
| F (1) | 1182.9 | 819.0 | 69.2 | 195.0 | 625.0 | 52.8 |
| G (1) | 1596.4 | 849.6 | 53.2 | 334.8 | 514.8 | 32.2 |

Several of the biomass samples described previously (table 4.4) were reanalyzed for protein content (of the alkali fractions) using the Ninhydrin assay (section 2.10.4.4). The concentrations of protein measured in samples digested with strong alkali were expressed in terms of ppm ($\mu g.ml^{-1}$) of carbon (column II; section 4.4.2). These values were then presented as a percentage (column III) of total carbon in the alkali fraction (column I). Analysis of the same samples, but not digested with strong alkali, resulted in concentrations of protein which were also expressed as concentrations of carbon (column IV). Subtraction of these values from those in column II gave the concentration of carbon derived from amino acids undetected by the assay (column V), *i.e.*, those amino acids present within whole proteins or peptide chains. These values were also presented as a percentage of the total carbon in the alkali fractions (column VI) to allow comparison with values obtained from alternative protein determination methods. x, number of samples used in analysis. Values from 2 or more samples within the same group were given as means plus/minus the standard deviations.

**Table 4.10. Efficiency of determination of protein in biomass fractions by the Ninhydrin assay.**

**a**

| fraction | A a-group (mM) | B a-group ($\mu g.ml^{-1}$) | C carbon (ppm) | D TOC (ppm) |
|---|---|---|---|---|
| COLD | 0.32 | 38.4 | 19.2 | 34.6 |
| HOT | 0.79 | 94.32 | 47.16 | 24.5 |
| ALK | 12.98 | 1557.24 | 778.62 | 1263.1 |
| RES | — | — | — | 23.7 |
| total protein | | 1.69mg.ml$^{-1}$ | | (2.69mg.ml$^{-1}$) |

**b**

| fraction | A a-group (mM) | B a-group ($\mu g.ml^{-1}$) | C carbon (ppm) | D TOC (ppm) |
|---|---|---|---|---|
| COLD | 1.72 | — | — | nd |
| HOT | 1.43 | — | — | nd |
| ALK | 11.92 | 1429.92 | 714.96 | 1232.1 |

**c**

| fraction | RNA (mM) | DNA (mM) |
|---|---|---|
| COLD | 0.044 | 0.015 |
| HOT | 0.065 | 0.024 |

**a** A standard solution of BSA (2.5mg.ml$^{-1}$) was subjected to fractionation method 1 (section 2.9.1) and each resulting fraction was assayed for free amino acids by the Ninhydrin method after alkali digestion (section 2.10.4.4). Determination of carbon in the fractions was by the TOCA (section 2.7.3). a-group, amino groups.

**b** A standard solution containing BSA (2.5mg.ml$^{-1}$), RNA (0.75mg.ml$^{-1}$) and DNA (0.25mg.ml$^{-1}$) was also subjected to the same fractionation method and the fractions were analyzed using the Ninhydrin assay and the TOCA. nd, not determined.

**c** Single standards of the same RNA and DNA concentrations were fractionated and assayed for amino groups.

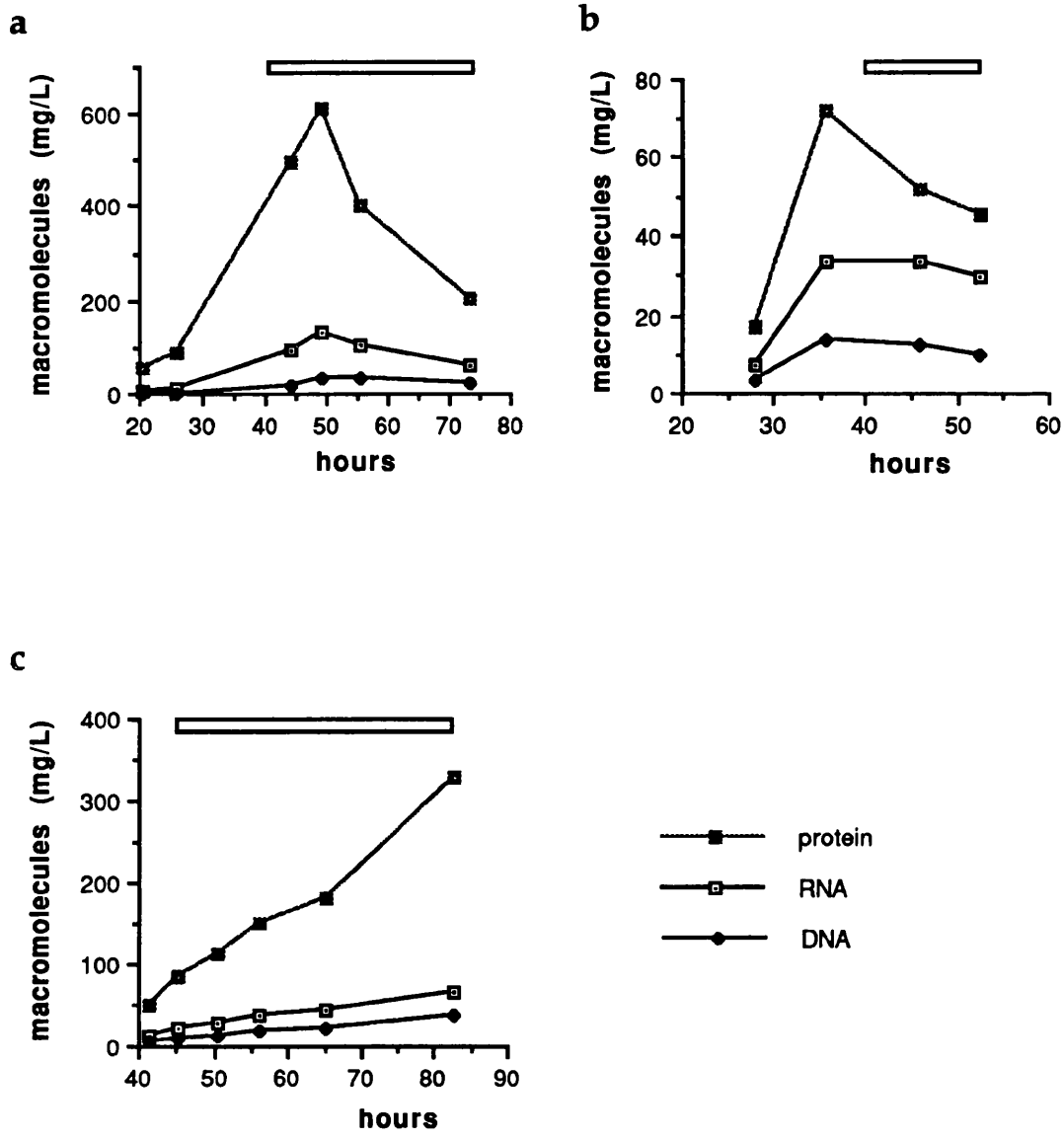



**Figure 4.3. Macromolecular change during fermentation of *S. coelicolor* in the Bioengineering fermenter.**
Samples taken during fermentations **a**, **b** and **c** described in section 3.5.1 (figure 3.6) were subjected to fractionation method 1 (section 2.9.1). The resulting hot PCA fractions were assayed for DNA and RNA by the Burton and orcinol methods respectively (sections 2.10.2 and 2.10.3). The protein concentrations of the alkali fractions were determined by carbon analysis (section 2.7.3). The values obtained were then expressed as per litre of fermenter cultures. The graphs show the change in macromolecular concentration in the cultures with time. The horizontal bars indicate the approximate periods of production of actinorhodin.

**Table 4.11. Percentage carbon in each fraction from *S. coelicolor* biomass grown in the fermenter.**

| a | hours | COLD | HOT | ALK | RES |
|---|---|---|---|---|---|
| | 20.25 | 7.4 | 12.5 | 61.9 | 18.1 |
| | 25.75 | 7.8 | 14.6 | 63.4 | 14.3 |
| | 44.25 | 10.2 | 17.0 | 59.5 | 13.4 |
| | 49.25 | 11.3 | 19.1 | 56.6 | 12.9 |
| | 55.25 | 10.0 | 21.8 | 55.9 | 12.3 |
| | 73.25 | 9.6 | 25.5 | 48.5 | 16.3 |

| b | hours | COLD | HOT | ALK | RES |
|---|---|---|---|---|---|
| | 28.0 | 5.2 | 25.4 | 42.3 | 27.1 |
| | 35.7 | 5.6 | 18.9 | 58.7 | 16.7 |
| | 45.8 | 5.3 | 23.4 | 53.0 | 18.2 |
| | 52.25 | 5.1 | 20.0 | 46.4 | 28.4 |

| c | hours | COLD | HOT | ALK | RES |
|---|---|---|---|---|---|
| | 41.5 | 10.3 | 22.8 | 52.9 | 14.0 |
| | 45.2 | 8.3 | 26.1 | 56.3 | 9.3 |
| | 50.5 | 12.1 | 21.1 | 49.4 | 17.4 |
| | 56.0 | 13.5 | 12.1 | 48.7 | 16.6 |
| | 65.2 | 11.1 | 20.6 | 48.4 | 19.9 |
| | 82.5 | 11.8 | 19.7 | 46.1 | 22.4 |

*S. coelicolor* 1147 was cultivated in three fermentations under the same conditions (sections 2.5.1, 2.6.2). Samples were removed from each fermentation at the stated times and washed biomass was subjected to fractionation method 1 (section 2.9.1). 10µl volumes of the resulting fractions (COLD, HOT, ALKALI and RESIDUE) were injected (5 times) into the TOCA (section 2.7.3) and the carbon concentration of each fraction was expressed as a percentage of the total carbon concentration of the biomass.

**Table 4.12. Quantitation of macromolecular concentrations in fermenter cultures of *S. coelicolor* 1147.**

| hours | CHO | RNA | DNA | PROTEIN |
|---|---|---|---|---|
| 22 (2) | 10.02 +/- 1.0 | 46.2 | 49.73 +/- 24.3 | 570.86 +/- 69.1 |
| 28 (3) | 7.04 +/- 2.8 | 121.09 +/- 78.1 | 52.82 +/- 32.3 | 528.06 +/- 148.6 |
| 35 (3) | 16.65 +/- 6.6 | 118.36 +/- 25.5 | 65.78 +/- 20.4 | 573.67 +/- 14.6 |
| 41 (3) | 10.93 +/- 11.7 | 112.02 +/- 31.3 | 54.57 +/- 11.8 | 424.17 +/- 91.2 |
| 45 (3) | 14.20 +/- 6.1 | 144.28 +/- 31.6 | 58.04 +/- 12.8 | 562.60 +/- 32.2 |
| 50 (5) | 15.47 +/- 13.9 | 119.70 +/- 29.7 | 53.58 +/- 10.2 | 526.89 +/- 43.1 |
| 56 (3) | 18.58 +/- 17.0 | 134.29 +/- 17.0 | 79.16 +/- 25.2 | 524.34 +/- 52.4 |
| 66 (2) | 14.18 +/- 12.6 | 118.12 +/- 1.6 | 47.88 +/- 9.6 | 139.12 +/- 63.8 |
| 73 | 15.71 | 149.42 | 97.6 | 485.86 |
| 82 | 15.84 | 93.62 | 51.2 | 461.27 |
| 93 | 13.9 | 105.84 | 36.81 | 392 |
| 108 | 6.32 | 81.24 | 26.61 | 445 |

28 *S. coelicolor* 1147 biomass samples from nine fermentations were analyzed for macromolecular content by fractionation method 1 (section 2.9.1) and the assays described in section 2.10. The macromolecular contents of the fractions were expressed as $mg.g^{-1}$ of dry weight and two or more samples harvested at similar times were tabulated as means plus/minus standard deviations. The bracketed values in the hours column indicate the number of samples used to obtain the means. A maximum of two or three samples were removed from some fermentations.

**Table 4.13. Comparison of two extraction methods.**

**a** Fractionation method 1

| fraction | TOC (ppm) | vol. (ml) | total TOC ($mg.ml^{-1}$) | % |
|---|---|---|---|---|
| COLD | 222.2 | 10.5 | 2.33 | 6.8 |
| HOT | 813.9 | 10.5 | 8.55 | 24.9 |
| ALK | 1676.4 | 10.5 | 17.60 | 51.3 |
| RES | 778.4 | 10.5 | 8.17 | 23.8 |
| total | | | 36.65 | |
| biomass | | | 34.30 | |

**b** Fractionation method 2

| fraction | TOC (ppm) | vol. (ml) | total TOC ($mg.ml^{-1}$) | % |
|---|---|---|---|---|
| 1 | 211.6 | 6.25 | 1.32 | 16.1 |
| 2 | 115.2 | 10.0 | 1.15 | 14.1 |
| 3 | 91.2 | 10.0 | 0.91 | 11.1 |
| 4 | 421.1 | 10.0 | 4.21 | 51.5 |
| 5 | 114.4 | 10.0 | 1.14 | 14.0 |
| total | | | 8.74 | |
| biomass | | | 8.17 | |

Three flasks containing 50ml of NMM-J were inoculated with *S. coelicolor* 1147 spores, incubated for 72 hours and biomass was harvested from the pooled cultures. Two portions of the biomass were subjected to the two fractionation methods described in section 2.9. A biomass concentration of 100mg (wet weight) per ml was used in fractionation method 1 (**a**) in a total volume of 10.5ml. Fractionation method 2 required a biomass concentration of 50mg (wet weight) per ml in a total volume of 5ml. To allow comparison of the two methods carbon analysis (section 2.7.3) was carried out on the resulting fractions and also on total biomass at the concentrations stated for both fractionation methods. The percentages of carbon present within each fraction were calculated with respect to biomass carbon (biomass). vol, volume.

**Table 4.14. Teichoic acid estimation by phosphate analysis.**

| fraction | phosphate (μmoles) | RNA (μmoles) | DNA (μmoles) | Total NA-P (μmoles) | TA-P (μmoles) |
|---|---|---|---|---|---|
| HOT (M1) | 58.26 | 22.647 | 8.361 | 31.008 | 27.252 |
| fraction 2 (M2) | 29.064 | 17.447 | 2.041 | 19.488 | 9.576 |
| fraction 3 (M2) | 14.196 | 2.495 | 5.636 | 8.131 | 6.065 |
| COLD (M1) | 88.77 | | | | |
| fraction 1 (M1) | 101.052 | | | | |

The fractions resulting from fractionation of *S. coelicolor* biomass grown in flasks by the two methods, as described in table 4.13, were further analyzed for their nucleic acid contents (sections 2.10.2, 2.10.3). The hot PCA fraction (method 1; M1) and fractions 2 and 3 from method 2 (M2) were analyzed in triplicate. These fractions were also subjected to phosphate determination (in triplicate; section 2.10.5). In addition, the phosphate contents of the cold PCA fraction (M1) and fraction 1 (M2) were measured. The combined RNA and DNA contents of the fractions were then expressed in terms of phosphate (total NA-P). An estimation of the teichoic acid content of the fractions was obtained from the teichoic acid phosphate (TA-P). This was calculated by subtracting the nucleic acid phosphate from total phosphate measured in the fractions.

**Table 4.15. Determination of efficiency of fractionation method 2 with respect to protein extraction.**

**a** BSA alone

| fraction | TOC (ppm) | protein ($\mu g.ml^{-1}$) | vol. (ml) | total protein ($\mu g$) |
|---|---|---|---|---|
| 1 | 20.6 | 41.2 | 6.25 | 257.5 |
| 2 | 20.5 | 41.0 | 6.25 | 256.25 |
| 3 | 14.6 | 29.2 | 5.0 | 146.0 |
| 4 | 541.6 | 1083.1 | 5.0 | 5415.5 |
| 5 | 16.6 | 33.2 | 5.0 | 166.0 |

**b** BSA + RNA + DNA

| fraction | TOC (ppm) | protein ($\mu g.ml^{-1}$) | total protein ($\mu g$) |
|---|---|---|---|
| 4 | 521.1 | 1042.3 | 5211.5 |
| 5 | 27.9 | 55.8 | 279.0 |

**a** A BSA standard of $1.25 mgml^{-1}$ was subjected to fractionation method 2 (section 2.9.2) using a total volume of 5ml (corresponding to a total of 250mg wet weight and 12.5mg dry weight; section 4.5.3). The resulting fractions were analyzed for carbon using the TOCA (TOC; section 2.7.3). The concentrations of carbon (as ppm) in each fraction were expressed in terms of concentrations of protein and the values were multiplied by the fraction volume to obtain the total amount of protein. vol, volume of fraction.
**b** A similar 5ml volume of BSA standard ($1.25 mg.ml^{-1}$) was also subjected to fractionation method 2 in combination with $0.375 mg.ml^{-1}$ RNA (total of 1.875mg) and $0.125 mg.ml^{-1}$ DNA (total of 0.625mg). The resulting fractions were measured for carbon content.

**Table 4.16. Reproducibility of fractionation method 2.**

| | fraction | TOC (ppm) | % | phosphate (μmoles) |
|---|---|---|---|---|
| I | 1 | 316.9 | 4.6 | 23.5 |
| | 2 | 1315.0 | 19.1 | 11.33 |
| | 3 | 785.0 | 11.4 | 3.98 |
| | 4 | 3418.0 | 49.5 | |
| | 5 | 1065.0 | 15.4 | |
| II | 1 | 360.0 | 4.9 | 28.0 |
| | 2 | 1348.0 | 18.3 | 9.2 |
| | 3 | 1440.0 | 19.6 | 4.1 |
| | 4 | 2937.0 | 39.9 | |
| | 5 | 1272.0 | 17.3 | |
| III | 1 | 348.8 | 5.3 | 29.9 |
| | 2 | 1538.0 | 23.3 | 11.3 |
| | 3 | 1297.0 | 19.6 | 2.9 |
| | 4 | 2438.0 | 36.9 | |
| | 5 | 987.0 | 14.9 | |

Triplicate biomass samples (I, II and III) obtained from pooled cultures of *S. coelicolor* 1147 grown in 50ml of NMM-J in flasks were subjected to fractionation method 2 (section 2.9.2). The resulting fractions were analyzed by the TOCA for carbon concentration (TOC; section 2.7.3) and the values were expressed as percentages of total carbon in the biomass. Phosphate analyses were also carried out on fractions 1, 2 and 3 from each sample.

**Table 4.17. Amino acid composition of biomass samples of *S. coelicolor* 1147.**

| | SAMPLES | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A | B | C | D | E | F | G | H |
| alanine | 0.244 | 0.250 | 0.477 | 0.590 | 0.266 | 0.552 | 0.528 | 0.652 |
| arginine | 0.132 | 0.111 | 0.211 | 0.378 | 0.126 | 0.239 | 0.217 | 0.243 |
| aspartate (+ asparagine) | 0.082 | 0.101 | 0.189 | 0.305 | 0.087 | 0.205 | 0.226 | 0.412 |
| cysteine | nd | nd | nd | nd | nd | nd | nd | nd |
| glutamate (+ glutamine) | 0.102 | 0.135 | 0.234 | 0.334 | 0.104 | 0.261 | 0.28 | 0.426 |
| glycine | 0.372 | 0.231 | 0.465 | 0.490 | 0.349 | 0.528 | 0.504 | 0.468 |
| histidine | 0.010 | 0.029 | 0.066 | 0.133 | 0.009 | 0.041 | 0.065 | 0.114 |
| isoleucine | 0.091 | 0.078 | 0.160 | 0.178 | 0.095 | 0.167 | 0.158 | 0.175 |
| leucine | 0.140 | 0.152 | 0.222 | 0.368 | 0.143 | 0.285 | 0.280 | 0.354 |
| lysine | 0.144 | 0.099 | 0.167 | 0.218 | 0.180 | 0.159 | 0.176 | 0.220 |
| methionine | 0.045 | 0.026 | 0.051 | 0.008 | 0.057 | 0.076 | 0.062 | nd |
| phenylalanine | 0.070 | 0.049 | 0.097 | 0.137 | 0.088 | 0.106 | 0.126 | 0.177 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| proline | 0.136 | 0.099 | 0.157 | 0.251 | 0.176 | 0.157 | 0.170 | 0.234 |
| serine | 0.103 | 0.078 | 0.162 | 0.196 | 0.108 | 0.180 | 0.166 | 0.163 |
| threonine | 0.143 | 0.085 | 0.153 | 0.299 | 0.134 | 0.179 | 0.203 | 0.191 |
| tryptophan | nd | nd | nd | nd | nd | nd | nd | nd |
| tyrosine | 0.044 | 0.024 | 0.046 | 0.089 | 0.061 | 0.074 | 0.054 | 0.073 |
| valine | 0.141 | 0.171 | 0.274 | 0.394 | 0.146 | 0.299 | 0.307 | 0.341 |



Eight biomass samples were harvested from four fermentations of *S. coelicolor* 1147 in NMM-J. The first four samples (A, B, C and D) were removed, one from each fermentation, before visible production of actinorhodin. Samples E, F, G and H were taken just after antibiotic production. Sample E corresponds to sample A with respect to the same fermentation and samples F, G and H correspond to samples B, C and D respectively. All samples were analyzed for amino acid content at a concentration of 50mg (wet weight) per ml. Biomass proteins were hydrolyzed in HCl for 24, 48 and 72 hours (section 2.12.2.1). Amino acids were identified and quantified by HPLC as described in section 2.12.2.5 and in appendix A. The resulting amino acid concentrations of each sample hydrolyzed for the different times were calculated as means plus/minus standard deviations (see appendix A) and then expressed as $mmol.g^{-1}$ dry weight (dry weight was determined by carbon analysis; section 2.7.3). The above table gives the mean values; the standard deviations from these means are tabulated in appendix A (table A3). All values are in $mmol.g^{-1}$ dry weight.

**Table 4.18. Amino acid composition of *S. coelicolor* 1147 expressed as percentages of the total amino acid content and comparison with the amino acid compositions of *E. coli* .**

| | SAMPLE (*Streptomyces coelicolor*) | | | | | | | | | (*E. coli*) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A | B | C | D | E | F | G | H | ord | a | b | c | ord |
| alanine | 12.2 | 13.5 | 15.5 | 14.8 | 13.4 | 14.7 | 13.5 | 15.6 | (1) | 9.4 | 9.6 | 12.7 | (1) |
| arginine | 6.6 | 6.0 | 6.7 | 6.6 | 6.3 | 6.0 | 8.6 | 5.8 | (6) | 5.2 | 5.5 | 5.4 | (7) |
| aspartate} | 4.1 | 5.5 | 5.8 | 5.9 | 4.4 | 6.3 | 7.0 | 9.8 | (7) | 4.2 | 4.5 | 9.9 | (12) |
| asparagine} | | | | | | | | | | 2.1 | 4.5 | | (13) |
| cysteine | nd | | | | | | | | | 2.1 | 1.7 | 1.7 | (17) |
| glutamate} | 5.1 | 7.3 | 7.3 | 7.3 | 5.2 | 7.8 | 7.6 | 10.2 | (5) | 7.3 | 4.9 | 10.5 | (5) |
| glutamine} | | | | | | | | | | 4.2 | 4.9 | | (10) |
| glycine | 18.6 | 12.5 | 14.8 | 14.5 | 17.6 | 14.0 | 11.2 | 11.2 | (2) | 8.9 | 11.4 | 8.0 | (2) |
| histidine | 0.5 | 1.6 | 1.2 | 2.1 | 0.4 | 1.8 | 3.0 | 2.7 | (15) | 1.0 | 1.8 | 1.0 | (18) |
| isoleucine | 4.5 | 4.2 | 4.7 | 5.0 | 4.8 | 4.4 | 4.1 | 4.2 | (12) | 5.2 | 5.4 | 4.6 | (8) |
| leucine | 7.0 | 8.2 | 8.0 | 6.9 | 7.2 | 7.8 | 8.4 | 8.5 | (4) | 8.4 | 8.4 | 7.9 | (3) |
| lysine | 7.2 | 5.3 | 4.4 | 5.2 | 9.1 | 4.9 | 5.0 | 5.3 | (8) | 8.4 | 6.4 | 7.0 | (4) |
| methionine | 2.2 | 1.4 | 2.1 | 1.6 | 2.9 | 1.7 | 0.2 | 0.05 | (16) | 4.2 | 2.9 | 3.4 | (14) |
| phenylalanine | 3.5 | 2.6 | 3.0 | 3.0 | 4.4 | 3.5 | 3.1 | 2.8 | (13) | 2.3 | 3.5 | 3.3 | (15) |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| proline | 6.8 | 5.4 | 4.4 | 4.9 | 8.9 | 4.8 | 5.7 | 5.6 | (9) | 5.2 | 4.1 | 4.6 | (11) |
| serine | 5.1 | 4.2 | 5.1 | 5.0 | 5.4 | 4.6 | 4.5 | 3.9 | (11) | 6.2 | 4.0 | 6.1 | (7) |
| threonine | 7.1 | 4.6 | 5.0 | 4.8 | 6.8 | 5.7 | 6.8 | 4.6 | (10) | 5.2 | 4.7 | 4.7 | (9) |
| tryptophan | nd | | | | | | | | | 1.0 | 1.1 | 1.0 | (19) |
| tyrosine | 2.2 | 1.3 | 2.1 | 1.4 | 1.5 | 3.1 | 2.0 | 1.7 | (14) | 2.1 | 2.6 | 2.1 | (16) |
| valine | 7.0 | 9.3 | 8.4 | 8.5 | 8.6 | 7.4 | 9.0 | 8.1 | (3) | 6.2 | 7.9 | 5.5 | (6) |



The amino acid contents of *S. coelicolor* 1147 biomass samples (A to H) given in table 4.17 were expressed as percentages of the total amount of amino acids detected. Amino acid compositions of *E. coli* expressed in the same manner were tabulated under the columns headed a, b and c. The figures in brackets denote the position of the amino acids in the order (ord) of decreasing relative proportions. a, from Holms (1986); b; from Neidhardt (1987); c, from Umbarger (1977).

**Table 4.19. Amino acid composition of the residue fractions of *S. coelicolor* biomass samples.**

| | SAMPLE | | | | |
|---|---|---|---|---|---|
| AMINO ACID | A | B | C | E | F |
| ADA | 0.032 | 0.036 | 0.052 | 0.050 | 0.050 |
| alanine | 0.064 | 0.104 | 0.136 | 0.108 | 0.106 |
| arginine | 0.004 | 0.017 | 0.018 | 0.008 | 0.016 |
| aspartate | 0.014 | 0.028 | 0.044 | 0.018 | 0.030 |
| cysteine | nd | | | | |
| glucosamine | 0.027 | 0.049 | 0.061 | 0.064 | 0.056 |
| glutamate | 0.054 | 0.050 | 0.110 | 0.050 | 0.052 |
| glycine | 0.046 | 0.084 | 0.109 | 0.076 | 0.098 |
| histidine | — | — | — | — | — |
| isoleucine | 0.004 | 0.012 | 0.016 | 0.008 | 0.012 |
| leucine | 0.008 | 0.027 | 0.034 | 0.016 | 0.028 |
| lysine | — | 0.005 | — | — | 0.01 |
| methionine | 0.004 | 0.008 | 0.010 | 0.008 | 0.012 |
| phenylalanine | 0.008 | 0.005 | 0.022 | 0.038 | 0.034 |
| proline | — | — | — | — | — |
| serine | 0.004 | 0.012 | 0.012 | 0.010 | 0.010 |
| threonine | — | 0.010 | 0.010 | 0.006 | 0.008 |
| tryptophan | nd | | | | |
| tyrosine | — | 0.005 | — | 0.002 | 0.004 |
| valine | 0.008 | 0.002 | 0.032 | 0.014 | 0.026 |

The residue fractions obtained from fractionation of samples A, B, C, E and F (see table 4.17) were subjected to acid hydrolysis (section 2.12.2.1) and were analyzed by HPLC for amino acid content. The solvent method used was method 3 (section 2.12.2.5). All figures are in $mmol.g^{-1}$. ADA, α-aminoadipate; glucosamine, N-acetylglucosamine; nd, not determinable.

**Table 4.20. Monomeric compositions of biomass samples of *S. coelicolor* 1147.**
**a, sample C.**

| protein amino acids | mmole.g$^{-1}$ | RNA nucleotides | mmole.g$^{-1}$ |
|---|---|---|---|
| alanine | 0.477 | AMP | 0.076 |
| arginine | 0.211 | GMP | 0.114 |
| aspartate} | 0.189 | CMP | 0.092 |
| asparagine} | | UMP | 0.067 |
| [x]cysteine | 0.064 | | |
| glutamate} | 0.234 | DNA nucleotides | |
| glutamine} | | dAMP | 0.025 |
| glycine | 0.465 | dGMP | 0.067 |
| histidine | 0.066 | dCMP | 0.067 |
| isoleucine | 0.160 | dTMP | 0.025 |
| leucine | 0.222 | | |
| lysine | 0.167 | Lipid components | |
| methionine | 0.051 | glycerol | 0.064 |
| phenylalanine | 0.097 | (glucose) | 0.009 |
| proline | 0.157 | ethanolamine | 0.033 |
| serine | 0.162 | av. fatty acid($C_{16}$) | 0.167 |
| threonine | 0.153 | | |
| [x]tryptophan | 0.032 | Peptidoglycan components | |
| tyrosine | 0.046 | N-acetyl- | |
| valine | 0.274 | glucosamine | 0.061 |
| | | alanine | 0.136 |
| | | glutamate | 0.110 |

Biomass samples of *S. coelicolor* 1147 harvested from four fermentations were analyzed for monomeric content using several methods. Amino acid analyses of total biomass and the resulting residue fractions after extraction (section 2.9.1) were carried out by HPLC (section 2.12). The contents of the amino acids assumed to be associated with peptidoglycan in the residue fraction were subtracted from total amino acid content and tabulated under the heading "peptidoglycan components". The nucleotide contents of the biomass were determined from the amounts of RNA and DNA using the appropriate calculations given in section 4.4.2. The lipid components were determined from total lipid of *S. lividans* (Grafe *et al.*, personal communication) using the equations in appendix C. x, these values were calculated assuming similar content to *E. coli.*
a Sample C as described in table 4.17 and section 4.6.2.

**Table 4.20** continued.
**b, Monomeric composition of *S. coelicolor* biomass sample D.**

| protein amino acids | mmole.g$^{-1}$ | RNA nucleotides | mmole.g$^{-1}$ |
|---|---|---|---|
| alanine | 0.590 | AMP | 0.090 |
| arginine | 0.378 | GMP | 0.135 |
| aspartate} | 0.305 | CMP | 0.108 |
| asparagine} | | UMP | 0.079 |
| $^{x}$cysteine | 0.210 | | |
| glutamate} | 0.334 | **DNA nucleotides** | |
| glutamine} | | dAMP | 0.026 |
| glycine | 0.490 | dGMP | 0.068 |
| histidine | 0.133 | dCMP | 0.068 |
| isoleucine | 0.178 | dTMP | 0.026 |
| leucine | 0.368 | | |
| lysine | 0.218 | **Lipid components** | |
| methionine | 0.008 | glycerol | 0.064 |
| phenylalanine | 0.137 | (glucose) | 0.009 |
| proline | 0.251 | ethanolamine | 0.033 |
| serine | 0.196 | av. fatty acid($C_{16}$) | 0.167 |
| threonine | 0.299 | | |
| $^{x}$tryptophan | 0.105 | **Peptidoglycan components** | |
| tyrosine | 0.089 | N-acetyl- | |
| valine | 0.394 | glucosamine | 0.085 |
| | | alanine | 0.152 |
| | | glutamate | 0.167 |

Sample D has been described previously in table 4.17 and section 4.6.2.

**Table 4.20** continued.
**c, Monomeric composition of *S. coelicolor* 1147 biomass sample A.**

| protein amino acids | mmole.g$^{-1}$ | RNA nucleotides | mmole.g$^{-1}$ |
|---|---|---|---|
| alanine | 0.244 | AMP | 0.050 |
| arginine | 0.132 | GMP | 0.077 |
| aspartate} | 0.082 | CMP | 0.069 |
| asparagine} | | UMP | 0.054 |
| [x]cysteine | 0.034 | | |
| glutamate} | 0.102 | DNA nucleotides | |
| glutamine} | | dAMP | 0.019 |
| glycine | 0.372 | dGMP | 0.044 |
| histidine | 0.010 | dCMP | 0.044 |
| isoleucine | 0.091 | dTMP | 0.019 |
| leucine | 0.140 | | |
| lysine | 0.144 | Lipid components | |
| methionine | 0.045 | glycerol | 0.064 |
| phenylalanine | 0.070 | (glucose) | 0.009 |
| proline | 0.136 | ethanolamine | 0.033 |
| serine | 0.103 | av. fatty acid($C_{16}$) | 0.167 |
| threonine | 0.143 | | |
| [x]tryptophan | 0.020 | Peptidoglycan components | |
| tyrosine | 0.044 | N-acetyl- | |
| valine | 0.141 | glucosamine | 0.027 |
| | | alanine | 0.064 |
| | | glutamate | 0.540 |

Sample C has been described previously in table 4.17 and section 4.6.2.

**Table 4.20** continued.
**d, Monomeric composition of *S. coelicolor* 1147 biomass sample B.**

| protein amino acids | mmole.g$^{-1}$ | RNA nucleotides | mmole.g$^{-1}$ |
|---|---|---|---|
| alanine | 0.250 | AMP | 0.051 |
| arginine | 0.111 | GMP | 0.077 |
| aspartate} | 0.101 | CMP | 0.069 |
| asparagine} | | UMP | 0.054 |
| [x]cysteine | 0.030 | | |
| glutamate} | 0.135 | DNA nucleotides | |
| glutamine} | | dAMP | 0.019 |
| glycine | 0.231 | dGMP | 0.044 |
| histidine | 0.029 | dCMP | 0.044 |
| isoleucine | 0.078 | dTMP | 0.019 |
| leucine | 0.152 | | |
| lysine | 0.099 | Lipid components | |
| methionine | 0.026 | glycerol | 0.064 |
| phenylalanine | 0.049 | (glucose) | 0.009 |
| proline | 0.099 | ethanolamine | 0.033 |
| serine | 0.078 | av. fatty acid($C_{16}$) | 0.167 |
| threonine | 0.085 | | |
| [x]tryptophan | 0.018 | Peptidoglycan components | |
| tyrosine | 0.024 | N-acetyl- | |
| valine | 0.171 | glucosamine | 0.049 |
| | | alanine | 0.104 |
| | | glutamate | 0.050 |

Sample D has been described previously in table 4.17 and section 4.6.2.

# Chapter 5

# Determination of throughputs and fluxes through the central metabolic pathways of *S. coelicolor* to biomass and to antibiotic production

## 5.1 Introduction

The previous chapters have been concerned with growth of *S. coelicolor* in a glucose minimal medium (chapter 3) and determination of the chemical composition of biomass grown in that medium (chapter 4). This chapter deals with the metabolism of *S. coelicolor* required for biosynthesis of new biomass during growth in NMM-J. A description of this metabolism is derived from measurement of the fluxes through the central metabolic pathways.

According to Holms (1986), metabolic fluxes may be calculated if the monomeric composition of the organism is known in addition to the central and biosynthetic pathways (section 1.10). Unfortunately, the central and biosynthetic pathways utilized by *Streptomyces* are as yet not clear. However, enzymes have been purified from *Streptomyces* which are similar in function to enzymes in *E. coli* and enzyme activities for bacterial pathways have been detected (section 1.8.2). This, therefore, allows the assumption that central metabolic pathways and the metabolic intermediates used as precursors for monomer biosynthesis are similar to those in *E. coli.* The approximate compositions of *S. coelicolor* biomass samples were not determined as comprehensively as that of *E. coli* but the data for the enterobacterium were gathered from analyses carried out by several groups over 30 years. As stated in section 4.1, the inventory of *E. coli* is not yet complete and will become more accurate in time. This also applies to the determination of fluxes through the central pathways. For example, in the 1950's not all of the components and metabolic pathways of *E. coli* had been elucidated. However, Roberts *et al.* (1955) described how the available knowledge of the metabolism of *E. coli* could be used to calculate the flow of metabolites from the glucose substrate to the synthesis of biomass (using glutamate, aspartate, pyruvate, serine and the Krebs Cycle intermediates as precursors). At that time, the proportion of the flow to biomass was reported to be 30% (with 45% to $CO_2$ and 25% excreted). 30 years later, the measured proportion of flow to biomass has been reported to be more than double that value, *i.e.*, 63% (Holms, 1986).

The determined monomeric composition of *S. coelicolor* samples (section 4.7) were sufficient for computation of approximate flux diagrams. In the Holms method of flux determination, the monomeric composition of 1g dry weight biomass is used to determine total amounts of intermediates in the central metabolic pathways required for biosynthesis of those monomers (*e.g.*, amino acids, nucleotides, etc.). As described in section 1.10, there are eight of these intermediates which act as precursors: glucose-6-phosphate (G6P), triose

phosphate (TP), phosphoglycerate (PG), phospho*enol*pyruvate (PEP), pyruvate (PYR), acetyl-CoA (AcCoA), oxaloacetate (OAA) and oxoglutarate (OGA; also known as α-ketoglutarate). Ingraham *et al.* (1983) provided a table (table 10 in the reference) showing the cost in metabolic precursors and in energetic terms for biosynthesis of each monomer in *E. coli* biomass. Erythrose-4-phosphate and ribose-5-phosphate were included as precursors but, for the purpose of flux determinations, Holms (1986) used glucose-6-phosphate as their precursor.

Tabulating the amount of each monomer present in 1g dry weight biomass with the amount of required precursors, allows calculation of the total amount of precursors needed for biosynthesis of whole biomass. These values represent the outputs from the central pathways and are connected to the input (glucose, in a glucose minimal medium) via throughputs (Holms 1986). Throughputs are calculated by subtracting from the input the amounts of precusors drained from central pathways. They are expressed as $mmol.g^{-1}$ and are a measure of the total amount of intermediates needed for 1g of biomass. The final throughput is to $CO_2$ and its magnitude is an indication of the efficiency of conversion of input to biomass output by the organism: a large amount of $CO_2$ produced implies that less carbon is converted to biomass. Estimation of efficiency of metabolism by this method is described later.

Metabolic fluxes are dynamic and dependent on the growth rate of the organism; the higher the growth rate, the faster the flux. Fluxes are, therefore, calculated by multiplying throughputs by the growth rate to give units of $mmol.g^{-1}.h^{-1}$. Growth of the organism on different substrates is likely to lead to differences in flux since metabolic adjustment occurs to allow for provision of all precursors for biosynthesis. For example, the flux through isocitrate dehydrogenase of *E. coli* grown on glucose was calculated to be equivalent to the flux through aconitase (a preceding enzyme in the TCA cycle). However, growth on acetate resulted in a flux through the dehydrogenase of two thirds that of aconitase (Holms, 1986). Growth of *E. coli* on acetate requires the enzymes of the glyoxylate bypass to provide precursors for biosynthsis by the, gluconeogenic route. This reduction in flux was indicative of the effects of regulation of activity of isocitrate dehydrogenase (Holms, 1986). Flux ratios are also important in the determination of regulation of excreted products. Comparison of fluxes before and during production of such metabolites would reveal alterations and, therefore, possible regulatory areas.

Flux determinations also provide information on the efficiency of conversion of input carbon to output carbon by the organism. According to Holms (1986), an

indicator of efficiency of utilization of glucose to provide biomass is given by the ratio of biosynthetic flux to the flux to $CO_2$. The efficiency of conversion of glucose carbon to biomass carbon by *E. coli* grown in glucose minimal medium was calculated to be 2.7 (Holms, 1986). Therefore, in addition to providing an indication of areas of regulation of fluxes, the Holms method of flux determination can give a measure of efficiency of growth of the organism concerned in the conditions used (Holms, 1986).

## 5.2 Objectives

This chapter is concerned with determination and comparison of fluxes to *S. coelicolor* biomass and to its excreted products, especially actinorhodin. This required attainment of the following objectives.

1. To determine the throughputs and fluxes through the central metabolic pathways of *S. coelicolor* to biomass. Throughputs would be calculated from the monomeric composition data from *S. coelicolor* biomass samples harvested prior to actinorhodin production (section 4.7) and using the assumption that central and biosynthetic pathways of *S. coelicolor* were similar to those of *E. coli*. As described in section 5.1, each monomer of biomass has precursors in the central metabolic pathways. It was therefore also assumed that these precursors were the same in both bacteria. Since *S. coelicolor* was grown on a glucose minimal medium, throughput diagrams could be constructed starting with a glucose input. Subtraction of the amounts of precursors required would give the throughputs. Fluxes could then be calculated from the throughputs and the growth rates during the fermentations from which the samples had been removed.

Determination of fluxes would also indicate how efficient *S. coelicolor* was in converting glucose carbon to biomass in NMM-J. Growth in flasks and in the fermenter had suggested that this efficiency was very low (sections 3.4, 3.5.1). The ratio of biosynthetic flux to $CO_2$ flux might support these observations.

2. To identify and quantify secondary metabolites excreted by *S. coelicolor* after cessation of growth to allow determination of fluxes to these metabolites. *S. coelicolor* produces the blue antibiotic actinorhodin (section 1.6.1). Although the actinorhodin biosynthetic pathway enzymes have not been characterized (although genetic analysis is extensive), it is known that the polyketide antibiotic is biosynthesised from 16 acetate molecules. Acetyl-CoA is therefore

the precursor from the central pathways required for biosynthesis of this molecule.

During conditions in which *S. coelicolor* produced another antibiotic, methylenomycin (section 1.6.5), excretion of two primary metabolites was observed to occur. These metabolites were pyruvate and α-ketoglutarate (Hobbs *et al.*, 1992). As described in section 1.8.2, excretion of keto-acids by *S. venezuelae* increased with mycelial age, coinciding with a reduction in enzyme activity. It would be of interest, therefore, to determine if *S. coelicolor* produced such keto-acids in batch cultivation, especially during production of actinorhodin.

3. To compare the fluxes through the central metabolic pathways of *S. coelicolor* to biomass and to actinorhodin production. To obtain biosynthesis of actinorhodin, alterations in fluxes to acetyl-CoA (or from acetyl-CoA) are likely to occur. Excretion of other metabolites would also require different fluxes than those to biomass. By comparing the ratio of flux to actionorhodin with those to biomass, it would be possible to identify these alterations in fluxes. This would result in identification of areas concerned with regulation of these fluxes. Areas involved in control of flux to actinorhodin production are important because deregulation of this flux could lead to increased productivity. Identification of such areas by flux determination would provide a foundation upon which further physiological and genetic studies could be based, thus contributing to a further understanding of the switch from primary to secondary metabolism in *Streptomyces*.

## 5.3 Determination of throughputs and fluxes through the central metabolic pathways of *S. coelicolor*; fluxes to biomass.

As described in section 5.1, computation of throughputs and fluxes by the Holms method (Holms, 1986) requires monomeric composition data. The data from *S. coelicolor* described in chapter 4 were therefore used to compute throughputs and fluxes specific to *S. coelicolor* grown in NMM-J prior to actinorhodin production. Calculations were simplified by use of the Excel spreadsheet programme (Microsoft).

### 5.3.1 Metabolic throughput estimations.

The total amounts of precursors required for biosynthesis of 1g dry weight *S. coelicolor* biomass are given in table 5.1a. The left hand column contains the monomeric composition of biomass sample C (described in table 4.20). The right hand columns contain the amounts of precursors needed to produce each monomer. For example, one mole each of pyruvate and oxaloacetate are required for biosynthesis of one mole of isoleucine, whereas leucine is formed from two moles of pyruvate and one mole of acetyl-CoA. The difference in biosynthesis of purines and pyrimidines is also in evidence since, in addition to glucose-6-phosphate, purines require phosphoglycerate while pyrimidines are biosynthesised from oxaloacetate. The total amount of each precursor needed is given in the bottom row. For biosynthesis of sample C the cost in pyruvate was the highest (table 5.1a). This was feasible because pyruvate is the precursor of several abundant amino acids in *S. coelicolor* including alanine. The second most required precursor was acetyl-CoA, mainly for synthesis of fatty acids. The order of requirement of these metabolites was opposite to that required for 1g of *E. coli* biomass (Holms, 1986). However, in both bacteria, triose phosphate was only used for fatty acid formation and was therefore required in the least amount. The order of the remaining metabolites was the same in the streptomycete and in the enterobacterium.

Additional precursor tables were obtained using the monomeric compositions of other samples previously described in section 4.: D, A and B (tables 5.1 b, c and d). The total amounts of precursors required for biosynthesis of sample D (table 5.1b) were slightly higher than those needed for sample C (table 5.1a) reflecting slight differences in macromolecular composition. Nevertheless, the order of precursor requirement was the same for the two samples. The total amount of triose phosphate was determined from the lipid composition of *S. livdans* (section 4.6.1) and so remained constant in all precursor tables regardless of sample content. This also applied to the amount of acetyl-CoA required for fatty acid biosynthesis.

Samples A and B (tables 5.1c and d) needed much less amounts of precursors for synthesis of their monomers in comparison to samples C and D. This was possibly due to the lack of material used for composition determinations caused by large water contents of the samples (section 3.5.1). However, carbon analyses had been carried out for dry weight measurements and would have removed any discrepancies caused by using wet weight measurements. Samples A and B had been harvested from different fermentations but at

similar times and were determined to have very similar compositions, especially in nucleic acid content. Therefore, similar amounts of precursors were required for whole biomass production. The precursor requirement orders differed from those of samples C and D with the amount of acetyl-CoA as the greatest needed. However, this was probably due to the fatty acid content (see above). The precursor order of sample B (table 5.1d) was similar to that of *E. coli*, but the order of sample A was different to that of the other samples with alterations in the order of pyruvate, acetyl-CoA, oxaloacetate, phosphoglycerate and glucose-6-phosphate. Phosphoglycerate and glucose-6-phosphate were very close in amounts required.

Estimations of throughputs of *S. coelicolor* were carried out by subtracting the amounts of precursors drained from the central metabolic pathways for biosynthesis of 1g dry weight biomass from the amount of glucose utilized during production of this biomass. 14.590mmol.$g^{-1}$ of glucose and 17.673mmol.$g^{-1}$ of glucose were utilized during production of samples C and D respectively. *E. coli* grown in a continuously fed-batch culture required 8.82mmol.$g^{-1}$ of glucose (Holms, 1986). The values for *S. coelicolor* were high in comparison to *E. coli* but possibly reflected the slow growth of the streptomycete. The resulting throughput diagrams for *S. coelicolor* samples C and D are given in figures 5.1a and 5.1b respectively. The large arrows indicate the drain of precursors to biosynthesis. The figures in the boxes are the throughputs expressed as mmol.$g^{-1}$ dry weight biomass. Those after triose phosphate (TP) double because of action of aldolase and triose phosphate isomerase resulting in two moles of glyceraldehyde-3-phosphate from fructose 1,6-diphosphate. In addition to abbreviated versions of the glycolytic pathway and TCA cycle showing only the metabolic precursors, the diagrams also show the throughput of PEP carboxylase (PEPC) which allows drainage to occur from OAA and OGA. The amounts of each precursor and glucose required for each sample were slightly different. Therefore, the throughputs also differed but only by approximately 15%. The final throughput in both samples (to $CO_2$) was high, however, and possibly reflected the efficiency of *S. coelicolor* in converting glucose to biomass during both fermentations. These high values show that a large proportion of the glucose was evolved as $CO_2$ which was produced by the reactions of pyruvate dehydrogenase and two TCA cycle enzymes ( see section 5.3.2).

The inputs required for biosynthesis of samples A and B were 147mmol.$g^{-1}$ and 235mmol.$g^{-1}$ respectively. These values were an order of magnitude greater than those for samples C and D. Construction of throughput diagrams using

the data in tables 5.1c and 5.1d would have resulted in throughputs which were not significantly different between enzymes in the pathways. Therefore, the data of samples A and B were not used further. Those of samples C and D were, however, used to compute diagrams of the fluxes to biomass.

### 5.3.2 Computation of metabolic fluxes to biomass in *S. coelicolor*.

Metabolic fluxes (expressed as $mmol.g^{-1}.h^{-1}$) are functions of throughputs of the central pathways and of the growth rate of the organism (sections 1.10, 5.1). The growth rates of *S. coelicolor* during the fermentations from which samples C and D were harvested were estimated at approximately $0.115h^{-1}$ and $0.089h^{-1}$ respectively. Computation of the fluxes (by multiplying throughputs by growth rate) through glycolysis and the TCA cycle of samples C and D resulted in figures 5.2a and 5.2b. Fluxes through the pathways are given in boxes while those to biosynthesis are represented by large arrows. The large throughputs to $CO_2$ were reflected in each flux diagram by the large fluxes through the TCA cycle.

The throughputs of samples C and D had been shown to be different (section 5.3.1). The fluxes through central metabolic pathways (and from them) for biosynthesis of 1g dry weight of each *S. coelicolor* sample were, however, very similar. This was shown further by the ratio of fluxes (shown in brackets) through the pathways with respect to a flux through phosphofructokinase (this flux was chosen as unity because it was the smallest, apart from that through PEPC). These ratios allowed comparison of fluxes to products excreted during the fermentations (section 5.4.2).

Additional information obtained from the flux diagrams was the amount of $CO_2$ produced per gram dry weight biomass per hour. $CO_2$ is evolved by the reactions of pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. A small amount of $CO_2$ is also produced during flux from glucose-6-phosphate to pentose phosphate in the pentose phosphate pathway when ribose groups for nucleotides are formed. However, $CO_2$ is required for carboxylation of PEP in the formation of OAA via PEPC. Therefore fluxes to production of $CO_2$ during each of the fementations were $7.014mmol.g^{-1}.h^{-1}$ and $6.301mmol.g^{-1}.h^{-1}$ (samples C and D respectively).

Effluxes to biomass and $CO_2$ production were used to compare inputs and outputs of the system of *S. coelicolor* metabolism by expressing all values in

terms of carbon fluxes. This was achieved by multiplying the fluxes from each precursor by the number of carbon atoms in the molecules (table 5.2). The input (glucose) was then expressed as 100% and the carbon effluxes were calculated accordingly. The highest proportions of carbon flux in the fermentations of samples C and D (table 5.2a and 5.2b respectively) were to $CO_2$ (69.6% and 66.7%). The remaining proportion to biomass was comprised mostly of the fluxes from G6P and PYR. The flux from TP was the smallest but as it was necessary for provision of glycerol in fatty acids, was extremely important for the organism. This was also the case in the flux from TP in *E. coli* grown in glucose minimal medium (Holms, 1986), as shown in table 5.2a. However, comparison of carbon fluxes to biomass of *E. coli* and *S. coelicolor* showed an approximate 2 to 3 fold difference in magnitude, even though *E. coli* also excreted acetate during growth. From the carbon contents of the medium from which samples C and D were harvested, it was assumed that excretion of metabolites by *S. coelicolor* had not occurred up to the time of harvesting. The flux to $CO_2$ during growth of *E. coli* was much less (23.1%) than that in the *S. coelicolor* fermentations which suggested greater efficiency of the *E. coli* biosynthetic pathways. As described in section 5.1, an indicator of efficiency of carbon conversion was given by Holms (1986) as the ratio of flux into biosynthesis to flux into $CO_2$. The efficiency for *E. coli* metabolism using the data given in table 5.2a was estimated to be 2.7. Those for *S. coelicolor* samples C and D were 0.4 and 0.5 respectively.

Flux to $CO_2$ had been calculated from the diagrams showing flux to biosynthesis. However, experimental data was required to verify if 70% of carbon from the glucose input was evolved as $CO_2$ in the streptomycete fermentations. An attempt was therefore made to measure experimentally the flux to $CO_2$ produced during the fermentation from which sample C was obtained. Measurement of $CO_2$ in the effluent gas was performed during this one fermentation, and the determination of the amount of $CO_2$ produced serves as an example of the information and calculations required.

Measurement of $CO_2$ was as a percentage of the effluent gas as detected by the analyser (section 2.8). A chart recorder was not available, but manual readings were taken frequently. The extent of $CO_2$ production by *S. coelicolor* is shown in figure 5.3. The percentage values are expressed as 100 times that recorded from the $CO_2$ analyser. It appeared as though $CO_2$ was produced in two stages: an initial log-type phase which reached a constant value by approximately 41 hours, and another production phase observed after onset of actinorhodin synthesis. $CO_2$ production can be used as an indication of growth of an

organism: the more metabolically active the cells, the more $CO_2$ produced. It therefore appeared as though two periods of increased metabolism were occurring during this fermentation of *S. coelicolor*.

Since the percentage values of $CO_2$ were changing constantly with time, an estimate of the average amount of $CO_2$ produced over certain time periods (sections I to X; figure 5.3) were obtained by measuring the area of the graph in each section. The average values were then corrected with respect to a background value of 0.04% (table 5.3a). In addition to the evolved gas, $CO_2$ was also present in the culture medium as carbonate acording to the equation:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The total amount of produced $CO_2$ therefore depended on the cumulative amount evolved in the effluent gas and on the amount present in solution at the time of harvesting the sample. The equations and calculations required to estimate this amount were based on those given by Hamilton (1972) for cultures of *E. coli* grown at 27°C and corrected for *S. coelicolor* at 30°C (appendix D). The amounts of $CO_2$ evolved in effluent gas and present in solution during each time section are given in tables 5.3a and 5.3b respectively. Column headings in table 5.3b correspond to the calculations in appendix D.

Sample C was harvested from the fermenter at the end of section IV (40.25 hours). At this time, the total amount of $CO_2$ evolved as gas was 103.31mmoles and the amount present in solution (in a volume of 6.73l) was 0.46mmoles. The total amount of $CO_2$ produced by this time was therefore 103.77mmoles corresponding to 1.24g of carbon. This value was used to estimate the proportion of carbon from the glucose input used for biomass and $CO_2$ production during the fermentation, as follows:

$$\underline{\text{Total } CO_2} = 103.77\text{mmoles} = \underline{1.24\text{g carbon}}$$

Biomass = 131mg dry weight per litre, total volume = 6.73litres
therefore, $\underline{\text{biomass}} = 881.6\text{mg} = \underline{0.44\text{g carbon}}$

$$\underline{\text{glucose}} \text{ utilized} = 4.5\text{g} = \underline{1.8\text{g carbon}}$$

According to Holms (1986), input is equivalent to output, *i.e.*:

$$\text{glucose} \rightarrow \text{biomass} + CO_2$$
$$1.8\text{g carbon} \rightarrow (1.24\text{g} + 0.44)\text{g carbon}$$
$$\underline{1.8\text{g carbon}} \rightarrow \underline{1.68\text{g carbon.}}$$

*i.e.*, 93.3% recovery of carbon with 68.9% evolved as $CO_2$ and 24.4% converted to biomass. From the flux estimations , the percentage of carbon flux to *S. coelicolor* biomass was 30.4 % while that to $CO_2$ was 69.6% (table 5.2). Therefore the values obtained from flux determinations were similar to those from experimental measurements of $CO_2$. However, the measured volumes of $CO_2$ produced from this fermentation were very low and therefore subject to error. In addition, since only one set of measurements was made it was only possible to use them as an example of the processes required to show experimental carbon balances (between input and output) of a bacterial system.

## 5.4 Determination of throughputs and fluxes to products excreted by *S. coelicolor*.

The main aim of this chapter was to compare fluxes through the central metabolic pathways of *S. coelicolor* to biomass with those to actinorhodin. Although the biosynthetic pathway of actinorhodin is not biochemically characterized (genetic studies have been extensive, however) it is known that the molecule is formed from acetate and therefore has acetyl-CoA as a precursor. Comparison of these fluxes would possibly result in identification of areas involved in controlling the flux to antibiotic biosynthesis. Determination of fluxes to biomass was described in the preceding section (section 5.3.2). This section therefore deals with fluxes to products excreted by *S. coelicolor*, especially actinorhodin.

### 5.4.1 Products excreted by *S. coelicolor* after cessation of growth.

The blue antibiotic, actinorhodin, is reportedly produced by *S. coelicolor* after biomass accumulation has ceased in batch cultures, *i.e.*, during stationary phase. Growth rate at this time is effectively zero (section 1.5.1). Since fluxes are a function of growth rate (flux = throughput x growth rate; sections 1.10, 5.1.), there are no fluxes of metabolites to biosynthesis of biomass during stationary phase. However, flux to biosynthesis of actinorhodin does occur as do fluxes to biosynthesis of any other metabolites excreted at this time.

The amounts of actinorhodin produced in the two fermentations from which samples C and D were harvested (section 4.6.2) were determined (section 2.11) and expressed per gram dry weight of biomass. No visible production of undecylprodigiosin took place at this time and it was assumed that methylenomycin was not produced in the culture conditions where nitrate was used as a nitrogen source (Hobbs *et al.*, 1992). However, Hobbs *et al.* (1992) had observed excretion of two organic acids, pyruvate and α-ketoglutarate, simultaneously with methylenomycin production. The observations took place during growth of *S. coelicolor* in a glucose minimal medium containing alanine as the nitrogen source. It was possible that organic acid excretion also occurred during actinorhodin production in NMM-J. Carbon analysis (section 2.7.3) of the culture medium at the time of harvesting had revealed the presence of extra carbon not derived from a medium component or actinorhodin. This carbon was therefore likely to have been produced by *S. coelicolor* and, based on accounts of keto-acid excretion by other *Streptomyces* (section 1.8.2), may have been derived from pyruvate and α-ketoglutarate molecules.

An attempt was therefore made to determine if *S. coelicolor* produced such keto-acids during production of actinorhodin. Triplicate flask cultures of *S. coelicolor* 1147 were prepared with NMM-J and glass beads (section 2.5.1). The flasks were cultivated (section 2.6.1) for 88 hours and 1ml samples were removed (2ml during actinorhodin production) approximately every 12 hours. All samples were analyzed for glucose utilization (section 2.7.4.1), actinorhodin production (section 2.11) and organic acid production by HPLC (section 2.13). Initial determination of organic acids resulted in a very sharp peak at a retention time of 10.033 to 11.08 minutes using samples diluted 100 fold (figure 5.4a). However, this peak was found to co-elute with HCl at a concentration of 100mM. Analysis of neat samples revealed a very broad peak at the same retention time and two very small peaks corresponding to α-ketoglutarate and pyruvate standards. The large peak was assumed to be caused by the presence of a high concentration of anionic compounds in the medium. These compounds possibly caused a change in light refraction at the detection wavelength (210nm; section 2.13) which was recorded as an absorbance change. This peak was ignored in further determinations of organic acids.

Attempts were subsequently made to confirm that the two peaks co-eluting with α-ketoglutarate and pyruvate standards were indeed these compounds (figure 5.4a). Initially, this involved "spiking" the samples with a small volume of a known concentration of each standard. The result was an increase in size of the small peak corresponding to either the α-ketoglutarate or pyruvate

standard (figures 5.4b and 5.4c). This strongly supported the idea that the two organic acids were these metabolites. However, a more precise means of identification was obtained for the pyruvate peak. This was performed using lactate dehydrogenase (LDH), an enzyme specific for pyruvate in the presence of NADH:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{(D)-LDH}} \text{D-lactate} + \text{NAD}^+$$

Use of LDH (as described in section 2.13.2) resulted in HPLC traces lacking in a peak corresponding to pyruvate (figure 5.5). An additional tiny peak appeared in these traces (figure 5.5b) which eluted at a similar time to a lactate standard. The identity of this peak was verified by spiking the samples containing LDH with the lactate standard. A corresponding increase in peak area was observed (figure 5.5c).

A further attempt was made to specifically confirm that the peak assigned to α-ketoglutarate was indeed this compound. This was carried out based on the theory that hydrolysis of α-ketoglutarate would result in succinate. Hydrolysis was therefore carried out in 1N HCl (section 2.13.2). A slight decrease in α-ketoglutarate peak size was observed after hydrolysis but only a small perturbation was visible in the base line to suggest that another metabolite had been formed (figure 5.6). However, the tiny peak did run with a succinate standard. It is possible that the hydrolysed peak was α-ketoglutarate, but the assumption used for flux determinations that an excreted metabolite was α-ketoglutarate was mainly based on the HPLC elution profiles. Pyruvate, however, had been positively identified as an excreted organic acid.

Figure 5.7 shows the results of these determinations in relation to glucose utilization and actinorhodin production. The cultures in flasks 1 and 2 were very similar in extent of uptake and production but the culture in flask 3 exhibited faster metabolism. However, the period of production of actinorhodin was similar in each culture and coincided with a second phase of glucose utilization which was faster than the first (this had also been observed in earlier cultivations in flasks and in the fermenter; sections 3.4, 3.5.1).

Small HPLC peaks corresponding to α-ketoglutarate and pyruvate standards were visible on the HPLC traces of samples taken from the time of onset of production of actinorhodin (figure 5.8d, e). However, the areas of these peaks were not integrated and so no measurement could be made of the concentrations of the organic acids. The points on the graph (figure 5.7) were

therefore obtained from larger peaks resolved from samples taken later in the fermentations (figure 5.8e, f, g, h). These traces showed increasing concentrations of the two organic acids as the fermentation progressed, followed by subsequent re-assimilation, mainly of pyruvate. This pattern is reflected in figure 5.7. Interestingly, small peaks corresponding to α-ketoglutarate and pyruvate were observed on traces of samples taken before production of actinorhodin, *i.e.*, during the growth phase (figure 5.8 a, b, c, d). Although the areas of these peaks were not integrated, a pattern of excretion and subsequent re-assimilation, similar to that during production of actinorhodin, was evident.

Determination of such organic acid excretion was from three flasks. It should be noted, therefore, that it is not possible to extrapolate these observations to other cultures of *S. coelicolor* grown in NMM-J either in flasks or in the fermenter.

### 5.4.2 Flux to excreted products.

To determine the flux to actinorhodin, the amount of all of the carbon excreted by *S. coelicolor* during the period of production of actinorhodin was measured (section 2.7.3) in the culture supernatants of the fermentations providing samples C and D (section 4.6.2). Subtraction of the carbon derived from Tris, glucose and actinorhodin in the medium from the total carbon resulted in a value of carbon from an unidentified source. This was denoted excess carbon (xs C). Some excess carbon in the supernatants of samples taken from three flask cultures was identified as α-ketoglutarate and pyruvate (section 5.4.1). Therefore, it was possible that a proportion of the excess carbon in the two fermenter samples was derived from these molecules. However, it is also possible that production of these organic acids only occurred in the flask cultures measured and, therefore, were not excreted in the fermenter cultures. Determination of the proportion of the carbon flux that was directed to production of actinorhodin only required knowledge of the flux from acetyl-CoA, assuming no other products were formed from acetyl-CoA. However, identification of the additional carbon excreted would have given a more complete picture of the alterations in the fluxes during production of actinorhodin as compared to those during the formation of biomass. To give an example of how this could be carried out, it was assumed that the excess carbon excreted by *S. coelicolor* during the two fermentations was completely derived from a-ketoglutarate and pyruvate. It should be noted, however, that the

calculated amounts of organic acids are over-estimates as compared to the amounts excreted in the measured flask cultures. In addition, the excess carbon may not have been attributable to these molecules at all.

The amounts of glucose required by 1g dry weight of biomass for biosynthesis of actinorhodin and excretion of pyruvate and α-ketoglutarate were calculated for the fermentations providing samples C and D. The following table shows the amounts of glucose required and metabolites (measured as excess carbon, xsC) produced over a stated time period:

| ferm | glc | act | xsC | α-KGA | PYR | h |
|---|---|---|---|---|---|---|
| C | 7.20 | 19.98 | 15.58 | 3.12 | 5.19 | 11.5 |
| D | 19.95 | 62.57 | 81.58 | 8.15 | 13.58 | 13.25 |

All values (except h, hours) are in mmoles.$g^{-1}$ dry weight biomass.

The values were used to construct throughput diagrams (figures 5.9a and 5.9b respectively). 1 mole of actinorhodin is formed from 16moles of acetate which was expressed as the drain from acetyl-CoA. Analogous with the throughput diagrams for biomass production, the throughputs in the two fermentations differed. On computation of fluxes (by dividing the throughputs by the time interval of actinorhodin production; figures 5.10a and 5.10b), the values from fermentation C were found to be approximately two to three fold higher than fermentation D. This is different to the fluxes to biomass which were found to be similar in both fermentations (section 5.3.2). However, the ratios of fluxes with respect to phosphofructokinase were calculated to be very similar (figures in brackets).

Expression of the fluxes in terms of carbon fluxes and with respect to glucose input gave the percentage values shown below.

| | fermentation C | | fermentation D | |
|---|---|---|---|---|
| | flux | % | flux | % |
| glucose | 3.756 | 100 | 9.036 | 100 |
| actinorhodin | 0.052 | 1.4 | 0.150 | 1.7 |
| pyruvate | 1.353 | 36.0 | 3.075 | 34.0 |
| α-ketoglutarate | 1.355 | 36.1 | 3.075 | 34.0 |
| $CO_2$ | 0.996 | 26.5 | 2.736 | 30.3 |

(fluxes are expressed as $mmol.g^{-1}.h^{-1}$).

The metabolite of interest was actinorhodin but the flux from glucose carbon to the antibiotic was very small (1.4% to 1.7%). During biomass production carbon fluxes from PEP and TP had been estimated at 1.1% and 0.2% of the total input flux respectively. Therefore, although the flux to actinorhodin was small, it is unlikley that it was unimportant to *S. coelicolor*. Approximately 70% of the carbon flux was to the excretion of excess carbon (assumed as pyruvate and α-ketoglutarate). The fluxes to these unknown compounds were therefore major during secondary metabolism of *S. coelicolor*. The flux to $CO_2$ was almost 30% of the input flux. In total, the fluxes added up to 100% and not greater (as in biomass carbon flux total; section 5.) because the flux through the pentose phosphate pathway could not be determined. The $CO_2$ flux during biomass production had been approximately 70%. It was therefore possible that *S. coelicolor* was more efficient at excreting metabolites than producing biomass when growth had ceased.

## 5.5 Comparison of fluxes to biomass and to excreted metabolite production.

The ratios of fluxes through the central metabolic pathways to the assumed excreted products (given by the example in previous section; 5.4.2) were used to compare the fluxes to antibiotic biosynthesis with those to biomass production (table 5.4). The main differences in the fluxes were those through pyruvate dehydrogenase, citrate synthase, from α-ketoglutarate dehydrogenase onwards and through PEP carboxylase. The fluxes through most of these enzymes were decreased during secondary metabolism but that through PEPC was increased by four-fold. The lower fluxes through pyruvate dehydrogenase and α-ketoglutarate dehydrogenase reflected the assumed accumulation of pyruvate and α-ketoglutarate. Because actinorhodin was biosynthesized, less acetyl-CoA would have been available for production of citrate via citrate synthase. Finally, the drop in flux through the TCA cycle from α-ketoglutarate dehydrogenase (because of the excretion of excess carbon represented as α-ketoglutarate) would have caused a reduction in the amount of OAA produced via malate dehydrogenase. Therefore, increased flux through PEPC would have occurred to maintain the higher flux through the initial TCA enzymes.

## 5.6 Discussion.

This chapter described the use of the determined monomeric composition of *S. coelicolor* in the calculation of throughputs and fluxes through the central metabolic pathways during biomass production (*i.e.*, during growth). Using the Holms method (Holms, 1986), computations of throughputs of four *S. coelicolor* biomass samples were performed by initial construction of precursor tables showing the total amounts of intermediates in the central pathways required for biosynthesis of 1g dry weight biomass of each of the samples. In the two samples C and D (which were subsequently used to describe the fluxes) the main drain to biosynthesis was from pyruvate for the provision of several major amino acids in *S. coelicolor*. Acetyl-CoA was also required in high quantities, mostly for fatty acid biosynthesis. Remaining precursors were required in the order OAA>PG>OGA>PEP>TP. Apart from pyruvate and acetyl-CoA, this order was similar to the order of requirement for *E. coli* biomass. This suggests that, assuming the metabolism of the two bacteria was similar, the data obtained from compositional analyses of samples C and D were feasible. Samples A and B, however, required these precursor metabolites in a different order to samples C and D but this may have been because of the low values of monomers. Acetyl-CoA was required in the highest amount followed by pyruvate (the order of sample A was directly comparable to that of *E. coli*). However, the amount of acetyl-CoA needed was possibly misleading since the fatty acid composition of all *S. coelicolor* samples was assumed to be equivalent to the lipid content of *S. lividans* and was therefore a constant value. The order of requirement of sample B was completely different to the other samples possibly reflecting the differences in composition of the biomass caused by differences in growth of *S. coelicolor* between fermentations.

The data from samples C and D were used to construct throughput diagrams (the data from samples A and B would have resulted in insignificant differences in throughputs because of the magnitudes of the input values). The throughputs of sample D were slightly higher than those of sample C but this did not affect the final throughputs which were very large in both samples. This final throughput was to $CO_2$ and indicated production of large quantities of the gas from continuous cycling of the TCA cycle with very little drainage to biosynthesis. This cycling was more clearly shown in the flux diagrams and expression of the fluxes in terms of carbon revealed that almost 70% of the input (glucose) carbon had been oxidized to $CO_2$, mainly via the TCA cycle. In a similar glucose minimal medium, *E. coli* was shown to produce only 23.1% of the input carbon as $CO_2$ (Holms, 1986). Such high production of $CO_2$ by *S.*

*coelicolor* in NMM-J suggests that metabolism was mainly directed towards energy production and not to anabolism. This is possibly not typical of *Streptomyces* growing in a minimal medium, since Bushell and Fryday (1983) showed that $8g.l^{-1}$ glucose was utilized by a 900ml culture of *S. cattleya* to produce $3.5g.l^{-1}$ biomass (excluding the inoculum concentration of $0.4g.l^{-1}$) over a period of 25 hours. This is a carbon conversion of 52% from glucose to biomass (*S. cattleya* biomass contains 47.5% carbon; Bushell and Fryday, 1983) and 48% to $CO_2$. An approximation of the amount of carbon evolved as $CO_2$ was also calculated from the points on the graph of rate of evolution of $CO_2$ (Bushell and Fryday, 1983). The equations in appendix D were used for this (working backwards). This resulted in 122.89mmoles $CO_2$ evolved from the 900ml culture during the 25 hour period. This was equivalent to 1.48g of carbon from 7.2g of glucose, *i.e.*, a conversion to $CO_2$ of 51.2%. Therefore, in comparison to fermentations of *S. cattleya*, a higher proportion of carbon was used by *S. coelicolor* to provide energy when grown in NMM-J.

Energy provided by successive turns of the TCA cycle without resulting increase in biomass is termed maintenance energy. This term was used mathematically to explain why specific rates of substrate consumption of micro-organisms did not decline to zero at zero growth rate (Marr *et al.*, 1963; Pirt, 1965). Maintenance energy is therefore very important at slow growth rates (Chesbro, 1988) and is used to sustain cellular vitality (*e.g.*, maintenance of solute gradients, turnover of macromolecules etc.; Pirt 1965). In glucose minimal medium, the growth rate of *E. coli* is $1.03h^{-1}$ (Neidhardt, 1987) or in substrate-sufficient cultures is $0.94h^{-1}$ (Holms, 1986). The maximum growth rate reached by *S. coelicolor* in NMM-J was approximately $0.12h^{-1}$. It is possible, therefore, that central metabolic activity of *S. coelicolor* was mainly directed towards production of energy for maintenance. At low growth rates, *E. coli* biomass has a higher proportion of protein than biomass grown at higher growth rates (Dennis and Bremer, 1987). The energy cost for protein synthesis by *E. coli* has been calculated to increase with decreasing growth rate (Bulthuis *et al.*, 1989). Protein synthesis could therefore be one of many processes diverting metabolic energy produced during activity of the central pathways away from biomass production. The consequences of low growth rates are discussed further in chapter 6.

Experimental estimation of $CO_2$ production in one fermentation supported the hypothesis that the majority of glucose carbon was not consumed for biosynthesis. However, the amount of $CO_2$ produced by the biomass in the fermenter was so little that the values obtained may have been affected by large

errors. Hobbs *et al.* (1990) reported maximum $CO_2$ production by *S. coelicolor* grown in HMM of 0.8% in the effluent gas. The maximum dry weight biomass in HMM was $3g.l^{-1}$. In the fermentation described in this chapter, the maximum biomass produced was $0.44g.l^{-1}$; a 6.8 fold difference. The maximum $CO_2$ produced by *S. coelicolor* in NMM-J was 0.15%; a 5.3 fold difference to that measured by Hobbs *et al.* (1990). It is possible, therefore, that the measurements made during this fermentation in NMM-J were feasible. However, additional measurements are required to verify this.

Production of samples C and D required fluxes of metabolites to be very similar through the central metabolic pathways of *S. coelicolor*. This was interesting as their macromolecular and monomeric compostions had differed. However, these differences may have been within experimental error as could the differences in growth rate during the two fermentations (approximately 20%). Fluxes for samples A and B were not calculated because fluxes through the pathways would have not been significantly different after precursor drainage. However, since the inputs to these samples were 8 to 13 fold greater than those to samples C and D and since the required intermediates were 2 to 3 fold less, these fluxes would have been different to those of samples C and D. Therefore, it is not possible to say that the flux diagrams obtained from samples C and D are typical for *S. coelicolor*.

Assuming that the metabolism of the two bacteria are similar, the magnitudes of the biomass monomers and the required amounts of precursors of *S. coelicolor* are feasible in comparison to the data from *E. coli* biomass compositions. In addition, the growth rate of the streptomycete was very low and so a large proportion of the input carbon would have been utilized to provide maintenance energy. Lastly, the composition of *E. coli* is not complete and will possibly alter as more accurate means of determination are used. Therefore, the magnitude of fluxes (which are dependent on the composition) will change. This will also be applicable to *Streptomyces*.

In an attempt to identify possible areas involved in the regulation of fluxes to the production of secondary metabolites, fluxes to actinorhodin were calculated. This also required measurement of additional carbon excreted by *S. coelicolor* in NMM-J. Pyruvate and α-ketoglutarate had been detected previously in the medium of *S. coelicolor* cultures (Hobbs *et al.*, 1992) during methylenomycin production and *Streptomyces* have been reported to excrete organic acids. Therefore, it was of interest to establish if such metabolites were excreted simultaneously with actinorhodin. Consequently, a preliminary

investigation was directed towards keto-acids. Both pyruvate and α-ketoglutarate were detected in three flask cultures and HPLC peaks corresponding to these keto-acids were observed both during actinorhodin production and during growth. The concentrations, especially of pyruvate, produced during secondary metabolism in these cultures were much higher than during primary metabolism. Production in both periods was similar, however, with α-ketoglutarate produced first followed by initial reabsorption of pyruvate. It was difficult to hypothesize that reassimilation of pyruvate was for biosynthesis of actinorhodin since glucose utilization occurred simultaneously. Reabsorption of the keto-acids had occurred as methylenomycin concentrations decreased (Hobbs *et al.*, 1992). In the culture in which glucose and pyruvate were exhausted, actinorhodin concentration was the highest. This suggests that both carbon sources may have been used for production of the antibiotic. However, because organic acid excretion was only measured in three flask cultures, it is not possible to state that similar excretion took place in other cultures of *S. coelicolor* grown in NMM-J.

Calculation of the flux to actinorhodin after cessation of biomass production by *S. coelicolor* showed that the flux to the antibiotic was only 1.4% to 1.7% of the total input. Although this flux was very small, it was greater than the primary fluxes from PEP and TP. Both these fluxes are essential for living cells. Therefore, the flux to actinorhodin was possibly of importance to *S. coelicolor*. However, the size of the flux posed problems in the comparison of the fluxes to biomass with this flux because of the possibility of errors.

To allow comparison of the fluxes during production of actinorhodin with those during biomass production, a flux diagram was constructed which assumed that excess carbon excreted by these fermenter cultures during production of actinorhodin was completely derived from α-ketoglutarate and pyruvate. This is a major assumption. However, the diagram was used as an example for the purpose of discussion and to show that the Holms method for determination of fluxes (Holms, 1986) could be used to calculate alterations in such fluxes. The differences observed were reductions in the ratios of fluxes through the enzymes: pyruvate dehydrogenase, PEP carboxylase, citrate synthase and α-ketoglutarate dehydrogenase (plus successive enzymes in the TCA cycle). The decrease in fluxes through these enzymes supported the assumed excretion of pyruvate and α-ketoglutarate and the measured excretion of an acetate containing compound, since a relative reduction in flux through pyruvate dehydrogenase and α-ketoglutarate dehydrogenase would have

resulted in metabolite accumulation. The decreased flux through citrate synthase would have resulted in a reduction in the extent of oxidation of acetyl-CoA via the TCA cycle. Therefore, diversion of flux to actinorhodin biosynthesis would be possible. Alternatively, the drain from acetyl-CoA caused by activity of actinorhodin biosynthetic enzymes would have reduced the amount of acetyl-CoA available for production of citrate.

In contrast, the relative flux through PEPC, in the example, was increased almost 4 fold. It is likely that this increase was to compensate for the large reduction in flux through malate dehydrogenase. The assumed excretion of α-ketoglutarate depended upon this anaplerotic flux and, since the flux to excretion was almost 35% of the total input flux, a larger flux from PEP was required than had been needed during biomass production. Activity of PEPC has been shown to increase throughout cultivation of *S. coelicolor* in HMM (H Bramwell, manuscript in preparation). In addition, increased activity of PEPC in cultures of *Streptomyces* sp. C5 was only detected during stationary phase (Dekleva and Strohl, 1988b); the activity was three-fold greater than that during growth phase. It is therefore possible that activity of PEPC in *S. coelicolor* grown in NMM-J does change during cultivation as actinorhodin is produced.

Regulation of fluxes depends on metabolite concentrations and control of enzyme activity. The possible influence of control of glycolytic and TCA cycle enzymes on regulation of fluxes is discussed further in chapter 6. It is difficult to identify possible areas of regulation of flux to actinorhodin because the flux was estimated to be so small. However, in the attempt to increase flux to actinorhodin via acetyl-CoA (and therefore increase volumetric productivity; section 1.) simply by using knowlede of the central metabolic pathways, a possible approach would be to reduce the flux through the TCA cycle to α-ketoglutarate excretion. This would be achieved by reducing the flux through PEPC thus providing more PEP for pyruvate kinase activity (assuming the enzyme is not saturated during the fluxes described here). Another approach would be to increase the flux through pyruvate dehydrogenase by increasing the gene dosage. This would provide more acetyl-CoA for actinorhodin biosynthesis. However, possible pyruvate excretion would have to be prevented, perhaps by maintaining a better carbon and nitrogen balance. There are, therefore, several possible approaches which could be taken to increase the flux to acetyl-CoA and, subsequently, synthesis of actinorhodin.

**Table 5.1a. Amounts of precursors required for biosynthesis of sample C.**

| monomer | content | central metabolic precursors | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | G6P | TP | PG | PEP | PYR | OAA | OGA | AcCoA |
| alanine | 0.477 | | | | | 0.477 | | | |
| arginine | 0.211 | | | | | | | 0.211 | |
| aspartate} | 0.189 | | | | | | 0.189 | | |
| asparagine} | | | | | | | | | |
| cysteine | 0.064 | | | 0.064 | | | | | |
| glutamate} | 0.234 | | | | | | | 0.234 | |
| glutamine} | | | | | | | | | |
| glycine | 0.465 | | | 0.465 | | | | | |
| histidine | 0.066 | 0.066 | | | | | | | |
| isoleucine | 0.160 | | | | | 0.16 | 0.16 | | |
| leucine | 0.222 | | | | | 0.444 | | | 0.222 |
| lysine | 0.167 | | | | | 0.167 | 0.167 | | |
| methionine | 0.051 | | | | | | 0.051 | | |
| phenylalanine | 0.097 | 0.097 | | | 0.194 | | | | |
| proline | 0.157 | | | | | | | 0.157 | |
| serine | 0.162 | | | 0.162 | | | | | |
| threonine | 0.153 | | | | | | 0.153 | | |
| tryptophan | 0.032 | 0.064 | | | 0.032 | | | | |
| tyrosine | 0.046 | 0.046 | | | 0.092 | | | | |
| valine | 0.274 | | | | | 0.548 | | | |
| A | 0.059 | 0.059 | | 0.059 | | | | | |
| dA | 0.025 | 0.025 | | 0.025 | | | | | |
| G | 0.115 | 0.115 | | 0.115 | | | | | |
| dG | 0.067 | 0.067 | | 0.067 | | | | | |
| C | 0.101 | 0.101 | | | | | 0.101 | | |
| dC | 0.067 | 0.067 | | | | | 0.067 | | |
| U | 0.078 | 0.078 | | | | | 0.078 | | |
| dT | 0.025 | 0.025 | | | | | 0.025 | | |
| C16 FA | 0.167 | | | | | 0.299 | 0.019 | | 1.476 |
| glycero-phosphate | 0.064 | | 0.064 | | | | | | |
| carbohydrate | 0.249 | 0.249 | | | | | | | |
| total | | 1.059 | 0.064 | 0.957 | 0.318 | 2.095 | 1.010 | 0.602 | 1.698 |

**Table 5.1b. Amounts of precursors required for biosynthesis of sample D.**

| monomer | content | central metabolic precursors | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | G6P | TP | PG | PEP | PYR | OAA | OGA | AcCoA |
| alanine | 0.590 | | | | | 0.590 | | | |
| arginine | 0.378 | | | | | | | 0.378 | |
| aspartate} | 0.305 | | | | | | 0.305 | | |
| asparagine} | | | | | | | | | |
| cysteine | 0.210 | | | 0.210 | | | | | |
| glutamate} | 0.334 | | | | | | | 0.334 | |
| glutamine} | | | | | | | | | |
| glycine | 0.490 | | | 0.490 | | | | | |
| histidine | 0.133 | 0.133 | | | | | | | |
| isoleucine | 0.178 | | | | | 0.178 | 0.178 | | |
| leucine | 0.368 | | | | | 0.736 | | | 0.368 |
| lysine | 0.218 | | | | | 0.218 | 0.218 | | |
| methionine | 0.008 | | | | | | 0.008 | | |
| phenylalanine | 0.137 | 0.137 | | | 0.274 | | | | |
| proline | 0.251 | | | | | | | 0.251 | |
| serine | 0.196 | | | 0.196 | | | | | |
| threonine | 0.299 | | | | | | 0.299 | | |
| tryptophan | 0.105 | 0.210 | | | 0.105 | | | | |
| tyrosine | 0.089 | 0.089 | | | 0.178 | | | | |
| valine | 0.394 | | | | | 0.788 | | | |
| A | 0.090 | 0.090 | | 0.090 | | | | | |
| dA | 0.026 | 0.026 | | 0.026 | | | | | |
| G | 0.135 | 0.135 | | 0.135 | | | | | |
| dG | 0.068 | 0.068 | | 0.068 | | | | | |
| C | 0.108 | 0.108 | | | | | 0.108 | | |
| dC | 0.068 | 0.068 | | | | | 0.068 | | |
| U | 0.079 | 0.079 | | | | | 0.079 | | |
| dT | 0.026 | 0.026 | | | | | 0.026 | | |
| C16 FA | 0.167 | | | | | 0.299 | 0.019 | | 1.476 |
| glycero-phosphate | 0.064 | | 0.064 | | | | | | |
| carbohydrate | 0.197 | 0.197 | | | | | | | |
| total | | 1.366 | 0.064 | 1.215 | 0.557 | 2.809 | 1.308 | 0.963 | 1.844 |

**Table 5.1c. Amounts of precursors required for biosynthesis of sample A.**

| monomer | content | central metabolic precursors | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | G6P | TP | PG | PEP | PYR | OAA | OGA | AcCoA |
| alanine | 0.244 | | | | | 0.244 | | | |
| arginine | 0.132 | | | | | | | 0.132 | |
| aspartate} | 0.082 | | | | | | 0.082 | | |
| asparagine} | | | | | | | | | |
| cysteine | 0.034 | | | 0.034 | | | | | |
| glutamate} | 0.102 | | | | | | | 0.102 | |
| glutamine} | | | | | | | | | |
| glycine | 0.372 | | | 0.372 | | | | | |
| histidine | 0.010 | 0.010 | | | | | | | |
| isoleucine | 0.091 | | | | | 0.091 | 0.091 | | |
| leucine | 0.140 | | | | | 0.280 | | | 0.140 |
| lysine | 0.144 | | | | | 0.144 | 0.144 | | |
| methionine | 0.045 | | | | | | 0.045 | | |
| phenylalanine | 0.070 | 0.070 | | | 0.140 | | | | |
| proline | 0.136 | | | | | | | 0.136 | |
| serine | 0.103 | | | 0.103 | | | | | |
| threonine | 0.143 | | | | | | 0.143 | | |
| tryptophan | 0.020 | 0.040 | | | 0.020 | | | | |
| tyrosine | 0.044 | 0.044 | | | 0.088 | | | | |
| valine | 0.141 | | | | | 0.282 | | | |
| A | 0.050 | 0.050 | | 0.050 | | | | | |
| dA | 0.019 | 0.019 | | 0.019 | | | | | |
| G | 0.077 | 0.077 | | 0.077 | | | | | |
| dG | 0.044 | 0.044 | | 0.044 | | | | | |
| C | 0.069 | 0.069 | | | | | 0.069 | | |
| dC | 0.044 | 0.044 | | | | | 0.044 | | |
| U | 0.054 | 0.054 | | | | | 0.054 | | |
| dT | 0.019 | 0.019 | | | | | 0.019 | | |
| C16 FA | 0.167 | | | | | 0.299 | 0.019 | | 1.476 |
| glycero-phosphate | 0.064 | | 0.064 | | | | | | |
| carbohydrate | 0.016 | 0.016 | | | | | | | |
| total | | 0.556 | 0.064 | 0.699 | 0.208 | 1.340 | 0.710 | 0.370 | 1.616 |

**Table 5.1d. Amounts of precursors required for biosynthesis of sample B.**

| monomer | content | central metabolic precursors | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | G6P | TP | PG | PEP | PYR | OAA | OGA | AcCoA |
| alanine | 0.250 | | | | | 0.250 | | | |
| arginine | 0.111 | | | | | | | 0.111 | |
| aspartate} | 0.101 | | | | | | 0.101 | | |
| asparagine} | | | | | | | | | |
| cysteine | 0.030 | | | 0.030 | | | | | |
| glutamate} | 0.135 | | | | | | | 0.135 | |
| glutamine} | | | | | | | | | |
| glycine | 0.231 | | | 0.231 | | | | | |
| histidine | 0.029 | 0.029 | | | | | | | |
| isoleucine | 0.078 | | | | | 0.078 | 0.078 | | |
| leucine | 0.152 | | | | | 0.304 | | | 0.152 |
| lysine | 0.099 | | | | | 0.099 | 0.099 | | |
| methionine | 0.026 | | | | | | 0.026 | | |
| phenylalanine | 0.049 | 0.049 | | | 0.098 | | | | |
| proline | 0.099 | | | | | | | 0.099 | |
| serine | 0.078 | | | 0.078 | | | | | |
| threonine | 0.085 | | | | | | 0.085 | | |
| tryptophan | 0.018 | 0.036 | | | 0.018 | | | | |
| tyrosine | 0.024 | 0.024 | | | 0.048 | | | | |
| valine | 0.171 | | | | | 0.342 | | | |
| A | 0.051 | 0.051 | | 0.051 | | | | | |
| dA | 0.019 | 0.019 | | 0.019 | | | | | |
| G | 0.077 | 0.077 | | 0.077 | | | | | |
| dG | 0.044 | 0.044 | | 0.044 | | | | | |
| C | 0.069 | 0.069 | | | | | 0.069 | | |
| dC | 0.044 | 0.044 | | | | | 0.044 | | |
| U | 0.054 | 0.054 | | | | | 0.054 | | |
| dT | 0.019 | 0.019 | | | | | 0.019 | | |
| C16 FA | 0.167 | | | | | 0.299 | 0.019 | | 1.476 |
| glycero-phosphate | 0.064 | | 0.064 | | | | | | |
| carbohydrate | 0.016 | 0.215 | | | | | | | |
| total | | 0.730 | 0.064 | 0.530 | 0.164 | 1.372 | 0.594 | 0.345 | 1.628 |

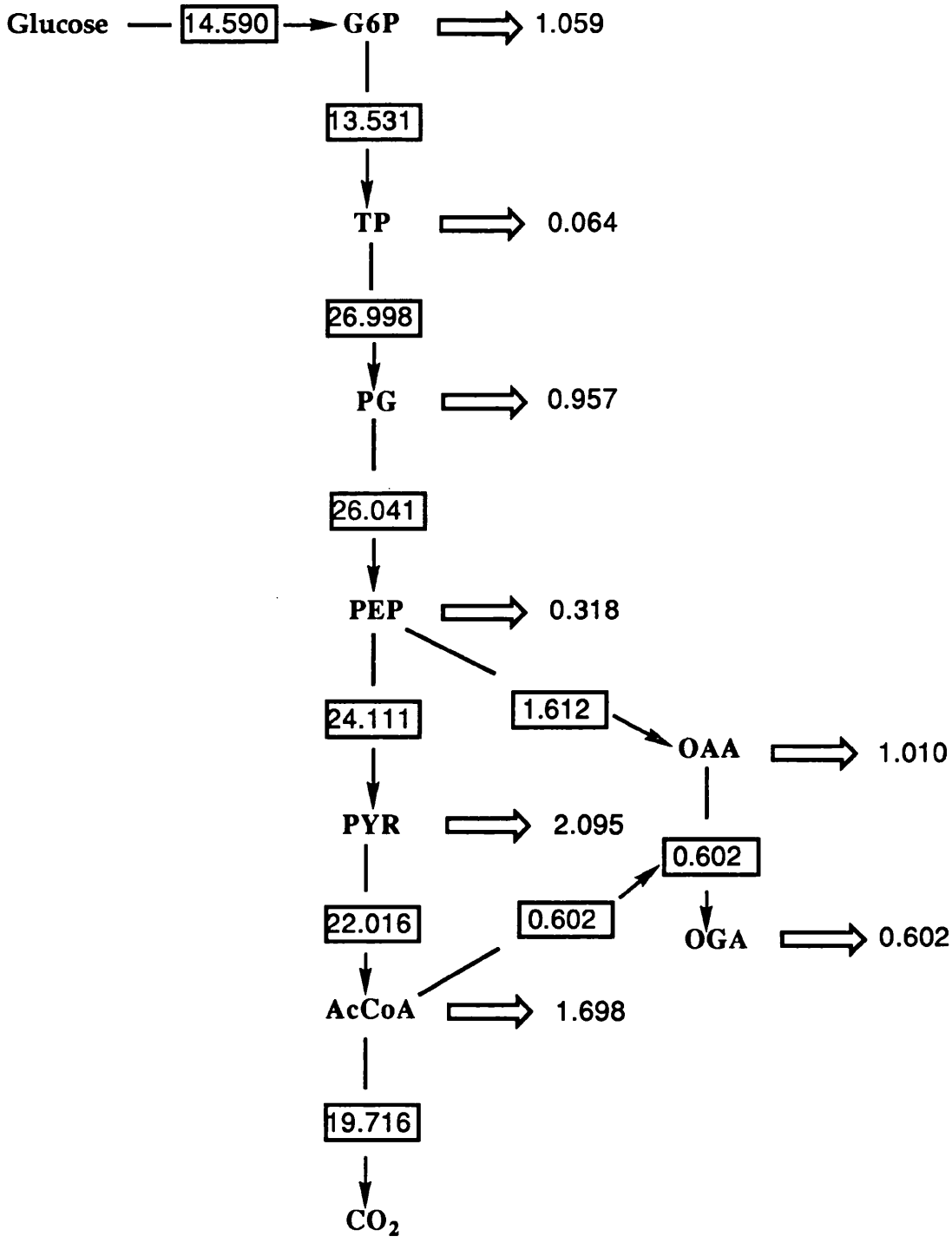



**Figure 5.1a. Diagram of throughputs from input to output (biomass) via the central metabolic pathways of *S. coelicolor* 1147. Sample C.**
The table was constructed using the data from table 5.1a. The drain from the intermediates (precursors) to biosynthesis are shown by wide arrows. Narrow arrows represent the throughputs of metabolites through glycolysis and the TCA cycle. The values were calculated by subtracting the amount of precursors required for biosynthesis of biomass from the input (glucose). All values are in $mmol.g^{-1}$ dry weight. G6P, glucose-6-phosphate; TP, triose phosphate; PG, phosphoglycerate; PEP, phospho*enol*pyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; OAA, oxaloacetate; OGA, oxoglutarate (α-ketoglutarate). The remaining flux to $CO_2$ is via pyruvate dehydrogenase and enzymes of the TCA cycle. This is shown in figure 5.2a.

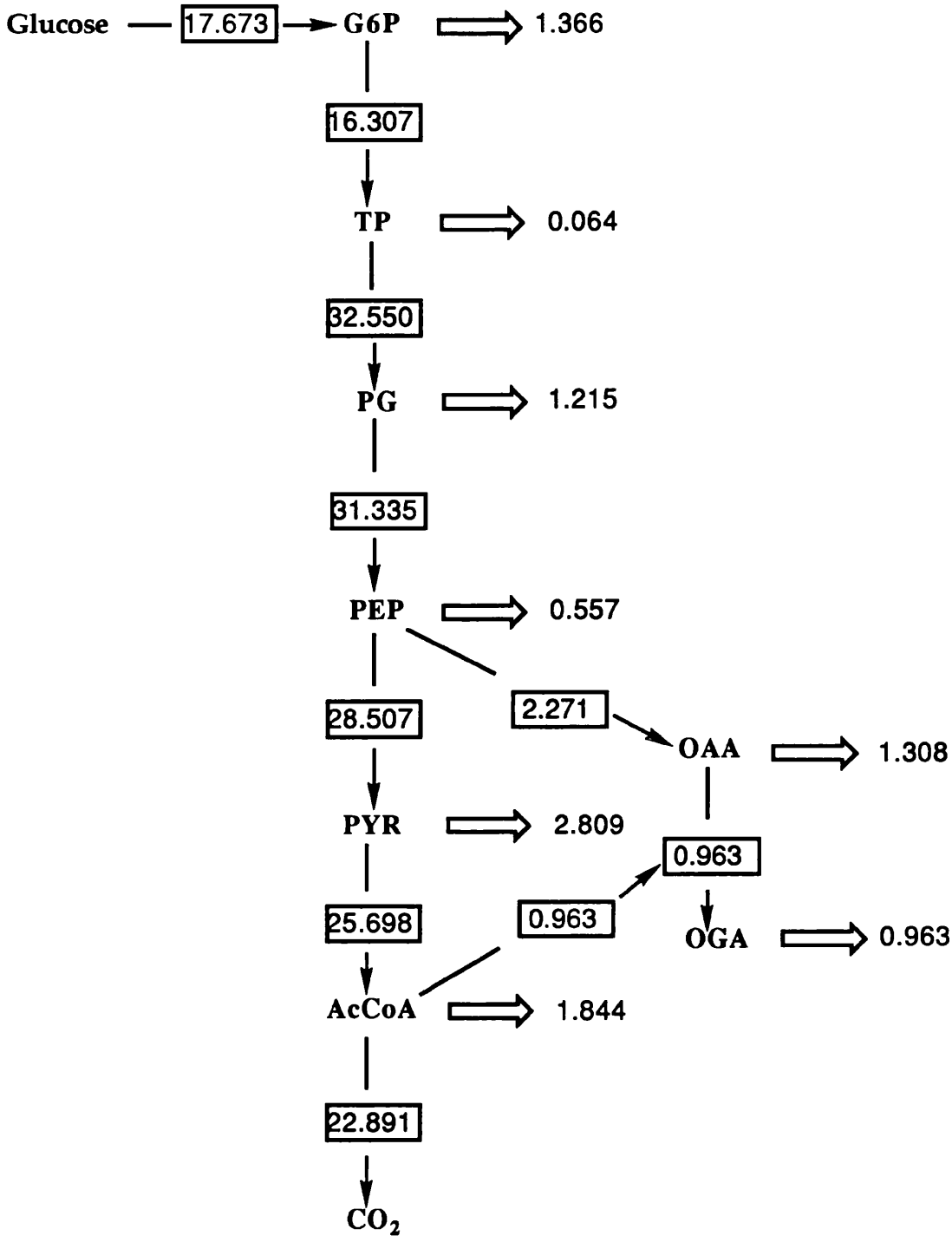



**Figure 5.1b. Throughput diagram. Sample D.**
The diagram was constructed using the data in table 5.1b. Nomenclature is the same as in figure 5.1a.

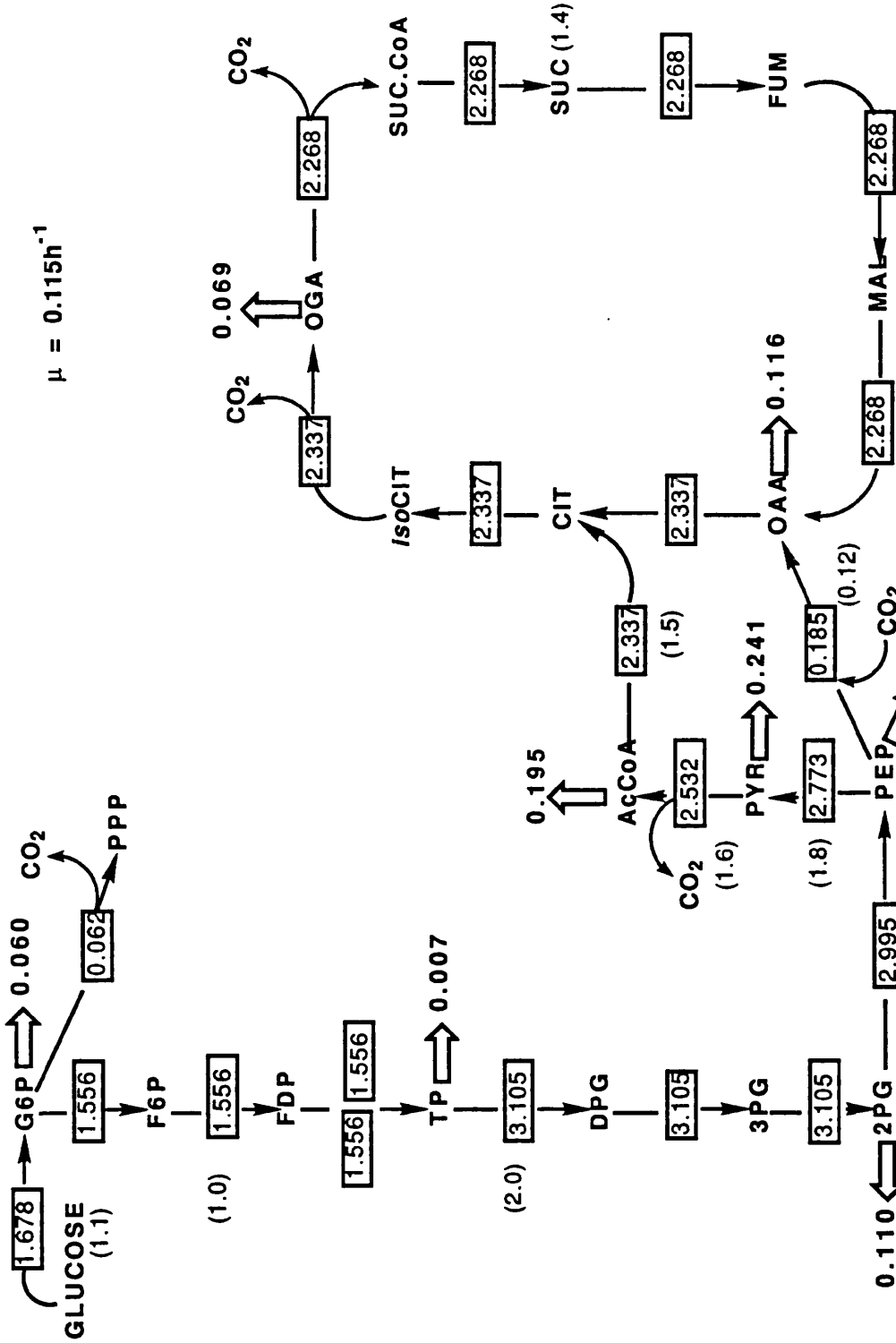



**Figure 5.2a. Fluxes through CMPs of *S. coelicolor* for production of 1g of biomass.** Sample C. All values are in $mmol.g^{-1}h^{-1}$. Figures in brackets are ratios of fluxes with respect to flux through PFK.

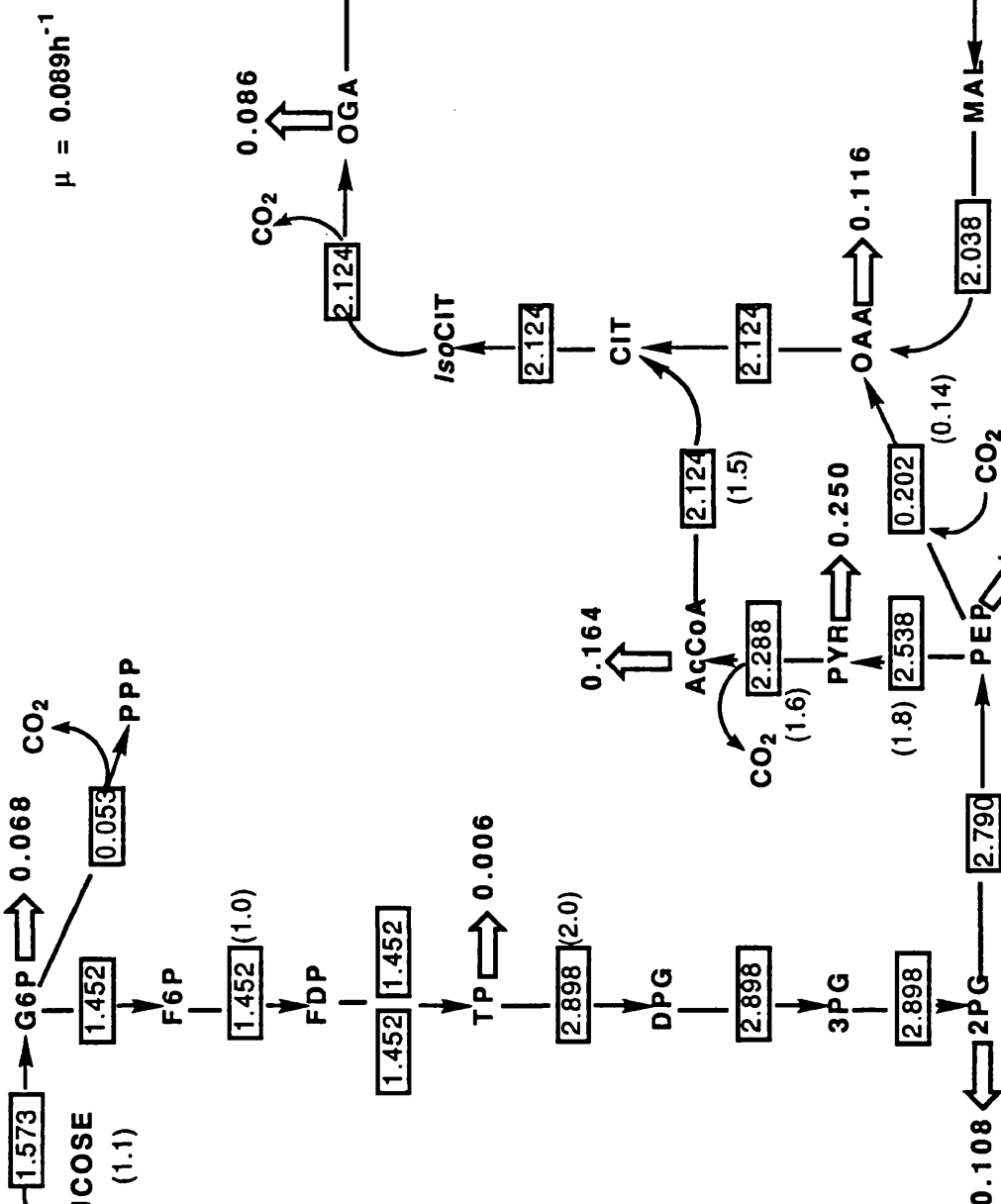



**Figure 5.2b. Diagram showing fluxes through the CMPs of *S. coelicolor* during production of 1g of biomass. Sample D.**

**Table 5.2. Flux of carbon to biomass and $CO_2$ from glycolysis and the TCA cycle.**

**a** Sample C.

| | | mmol.g$^{-1}$h$^{-1}$ | % | %$^{E}$ |
|---|---|---|---|---|
| Input | Glucose | 10.07 | 100 | 100 |
| Output | G6P | 0.73 | 7.2 | 17.6 |
| | TP | 0.02 | 0.2 | 0.6 |
| | PG | 0.33 | 3.3 | 6.0 |
| | PEP | 0.11 | 1.1 | 2.5 |
| | PYR | 0.72 | 7.1 | 10.3 |
| | AcCoA | 0.39 | 3.9 | 7.8 |
| | OAA | 0.46 | 4.6 | 10.1 |
| | OGA | 0.35 | 3.5 | 7.9 |
| | $CO_2$ | 7.01 | 69.6 | 23.1 |
| | | | 100.5 | 15.5[a] |
| | | | | 101.4 |

**b** Sample D.

| | | mmol.g$^{-1}$h$^{-1}$ | % |
|---|---|---|---|
| Input | Glucose | 9.44 | 100 |
| Output | G6P | 0.73 | 7.7 |
| | TP | 0.02 | 0.2 |
| | PG | 0.32 | 3.4 |
| | PEP | 0.15 | 1.6 |
| | PYR | 0.75 | 7.9 |
| | AcCoA | 0.33 | 3.5 |
| | OAA | 0.46 | 4.9 |
| | OGA | 0.43 | 4.6 |
| | $CO_2$ | 6.30 | 66.7 |
| | | | 100.6 |

Fluxes from each of the metabolic precursors in the central pathways of *S. coelicolor* to biosynthesis (figure 5.2) were expressed in terms of carbon flux, *i.e.*, the flux from each intermediate was multiplied by the number of carbon atoms present in their molecular structures. E, the relative amounts of carbon flux via intermediates in *E. coli* (from Holms, 1986); a, flux to acetate.

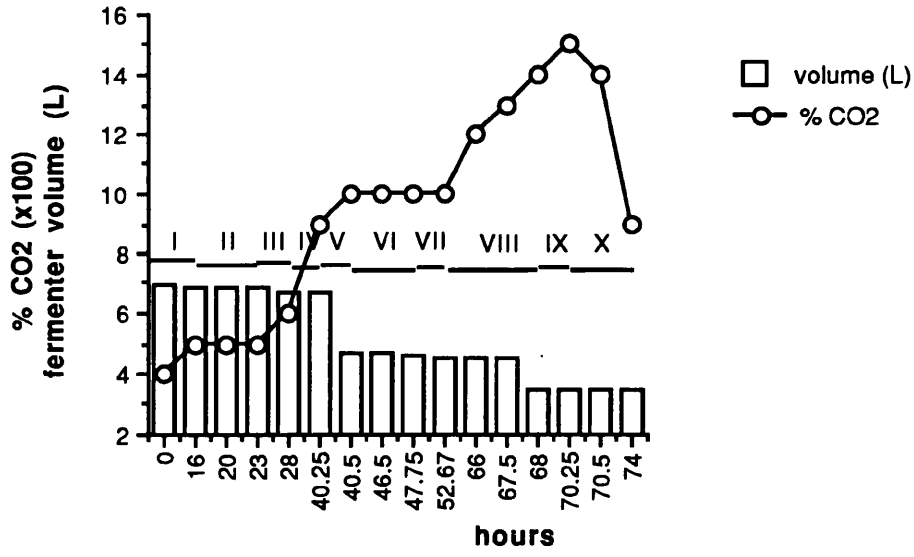



**Figure 5.3. Gaseous $CO_2$ production by *S. coelicolor* 1147 during a fermentation.**

Gaseous $CO_2$ production by *S. coelicolor* 1147 during growth in the fermenter was measured as a percentage of the effluent gas by means of a $CO_2$ analyzer (section 2.8). The flow rate of air through the fermenter was kept constant at 1volume.volume$^{-1}$.min$^{-1}$ by altering the flow after each sample was taken. The values on the graph are the values measured multiplied by 100. Average values over a certain time period (each time period is a section marked I to X) were calculated by measuring the area of the graph over that period. The time period (section) corresponded to the length of the graph, therefore dividing the area by the length gave the height, *i.e.*, the average % $CO_2$ produced. A measurement of the culture volume in the fermenter during each time period was required to calculate the total amount of $CO_2$ produced during the fermentation. The culture volume at certain times is given in terms of a bar chart. The volume was constant in each section.

**Table 5.3. Estimation of $CO_2$ produced by *S. coelicolor* 1147 in effluent gas and in solution.**

**a Gaseous $CO_2$.**

| section | average $CO_2$ (%) | corrected $CO_2$[x] (%) | $CO_2$ evolved (ml) | $CO_2$ evolved (mmol) |
|---|---|---|---|---|
| I | 0.046 | 0.006 | 403.20 | 16.22 |
| II | 0.050 | 0.010 | 293.16 | 11.79 |
| III | 0.05 | 0.010 | 209.40 | 8.42 |
| IV | 0.073 | 0.033 | 1662.68 | 66.88 |
| V | 0.095 | 0.055 | 55.52 | 2.23 |
| VI | 0.100 | 0.060 | 1234.53 | 49.66 |
| VII | 0.100 | 0.060 | 816.52 | 32.84 |
| VIII | 0.108 | 0.068 | 2808.33 | 112.96 |
| IX | 0.147 | 0.107 | 504.13 | 20.28 |
| X | 0.117 | 0.077 | 604.64 | 34.32 |
| | | | | 345.60 |

**b $CO_2$ in solution.**

| section | pH | $[CO_2]$ (mM) | $[HCO_3]^- + [CO_2]$ (mM) |
|---|---|---|---|
| I | 6.95 | 0.002 | 0.010 |
| II | 6.90 | 0.003 | 0.014 |
| III | 6.95 | 0.003 | 0.016 |
| IV | 7.10 | 0.010 | 0.069 |
| V | 7.10 | 0.016 | 0.111 |
| VI | 7.25 | 0.018 | 0.169 |
| VII | 7.25 | 0.018 | 0.169 |
| VIII | 7.50 | 0.020 | 0.318 |
| IX | 7.60 | 0.031 | 0.612 |
| X | 7.65 | 0.022 | 0.485 |

The calculations were carried out using the equations in appendix D. The sections refer to those in figure 5.3. x, the back-ground percentage value was subtracted from each measured value to give the corrected percentage value.

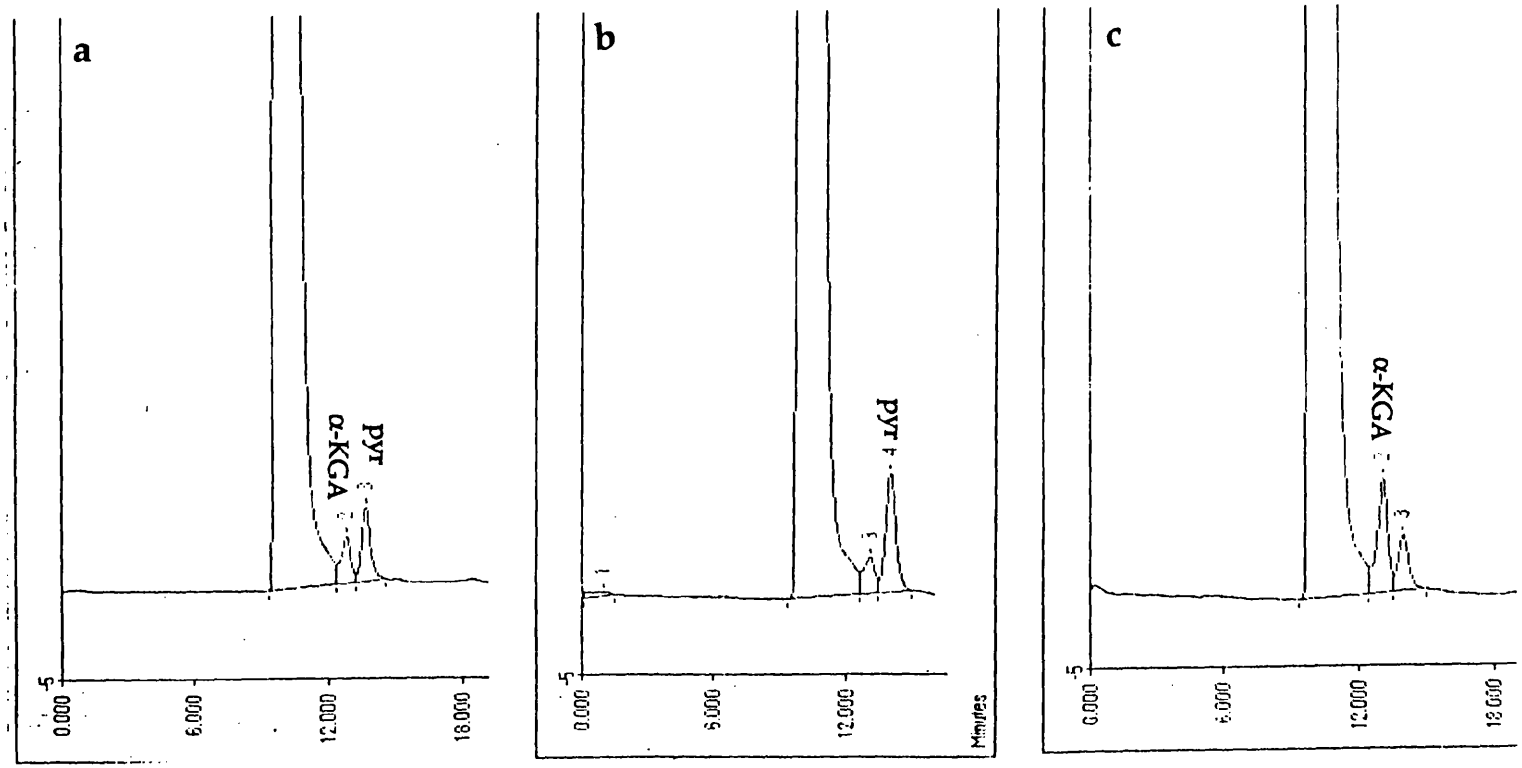



**Figure 5.4. HPLC trace of organic acids excreted by *S. coelicolor* in a flask culture during production of actinorhodin.** Triplicate flask cultures of *S. coelicolor* 1147 were incubated at 30°C over a period of 88 hours. 1ml samples were taken from the cultures on a 12 hourly basis (approximately); 2ml samples were taken from the time of onset of production of actinorhodin. Samples were spun in a microfuge at maximum speed for 5min. and the supernatants and biomass pellets were separated. Undiluted supernatants were then analyzed for organic acid excretion by *S. coelicolor* by HPLC (section 2.13.1). **a** Trace of undiluted sample taken at 51.4 hours after inoculation. Large peak is the result of the high concentration of anions in NMM-J. Small peaks corresponded to α-ketoglutarate and puruvate standards. **b** The same sample shown in figure 5.4 a was "spiked" with 5mM pyruvate in a 9 to 1 ratio and the product was analyzed by HPLC (section 2.13.1). **c** The same sample was "spiked" with 5mM α-ketoglutarate in the ratio 9 to 1, followed by analysis using the HPLC.

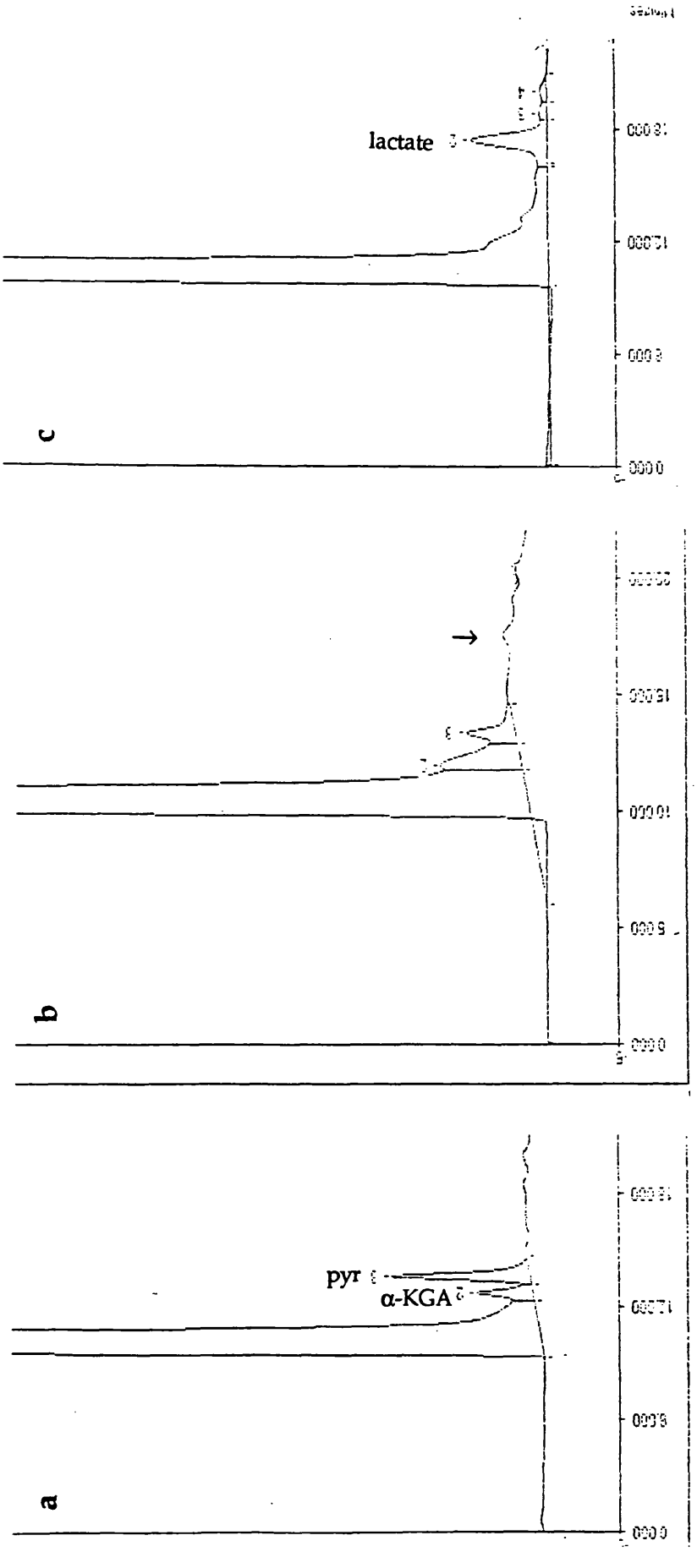



**Figure 5.5. HPLC trace showing positive identification of pyruvate peak using lactate dehydrogenase.** Samples from the triplicate flask cultures described in figure 5.4 were analyzed further for pyruvate excretion by positively identifying the peak corresponding to the pyruvate standard. This was carried out by analyzing the product obtained from the reaction of the pyruvate in the samples with NADH in the presence of lactate dehydrogenase (section 2.13.2). The resulting lacate peak was identified using the appropriate standard. **a** Trace of undiluted sample taken from a flask culture at 63.75 hours after inoculation. **b** Trace of sample after reaction of pyruvate and NADH in the presence of lactate dehydrogenase. Broad peak indicates the co-elution of α-ketoglutarate with the unreacted NADH. Arrow points to proposed lactate peak. **c** Lactate peak was identified by "spiking" reacted sample (figure 5.5b) with an authentic lactate standard.

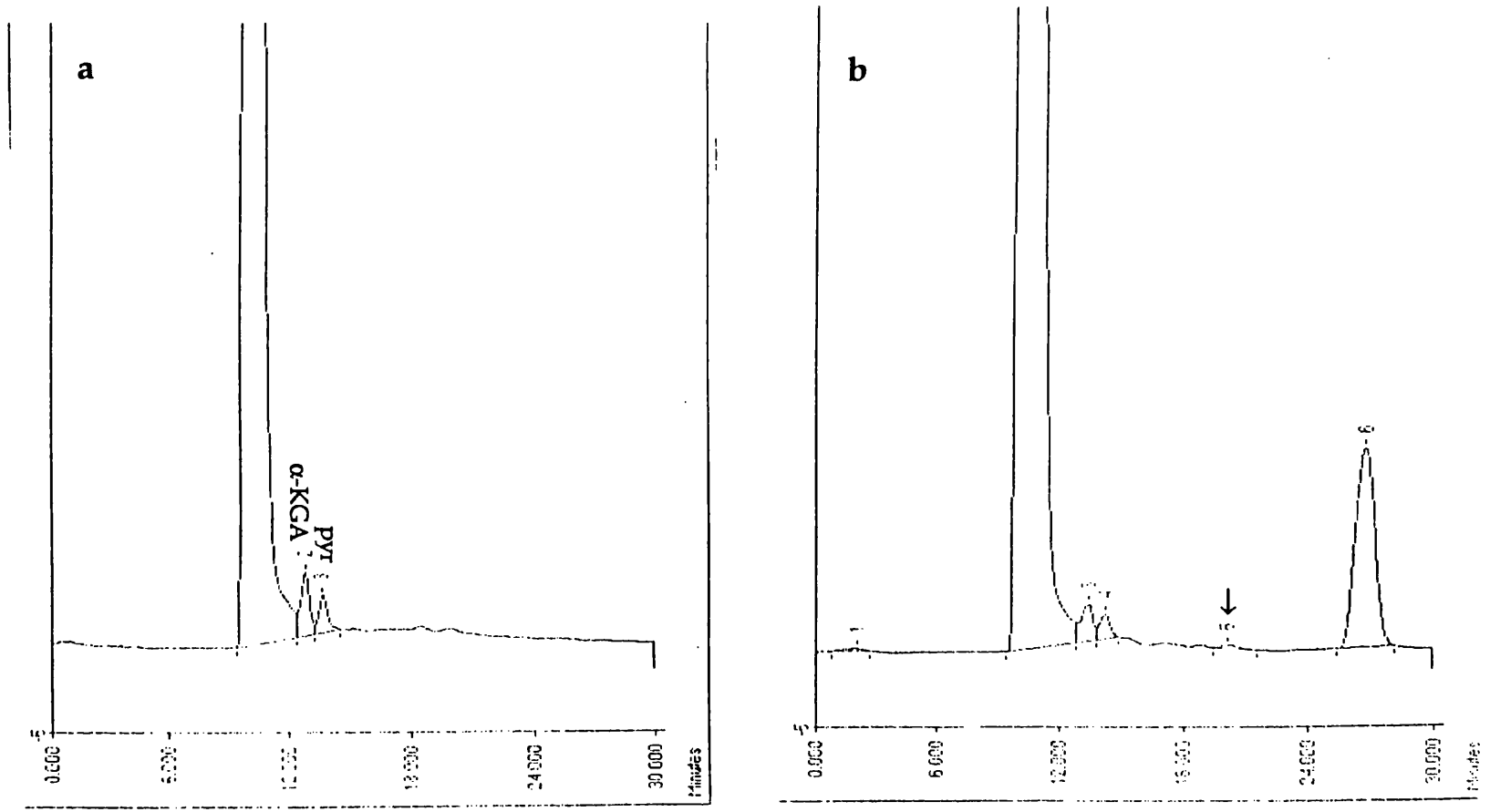



**Figure 5.6. HPLC traces showing possible acid hydrolysis of α-ketoglutarate to give succinate.** Samples taken from flask cultures, described in figure 5.4, at 63.75 hours after inoculation were hydrolyzed with 1N HCl in a boiling water bath (section 2.13.2). **a** Undiluted sample. **b** Sample after hydrolysis. The arrow points to a peak possibly corresponding to succinate. The large peak, which was eluted late in the HPLC run, was not identified.

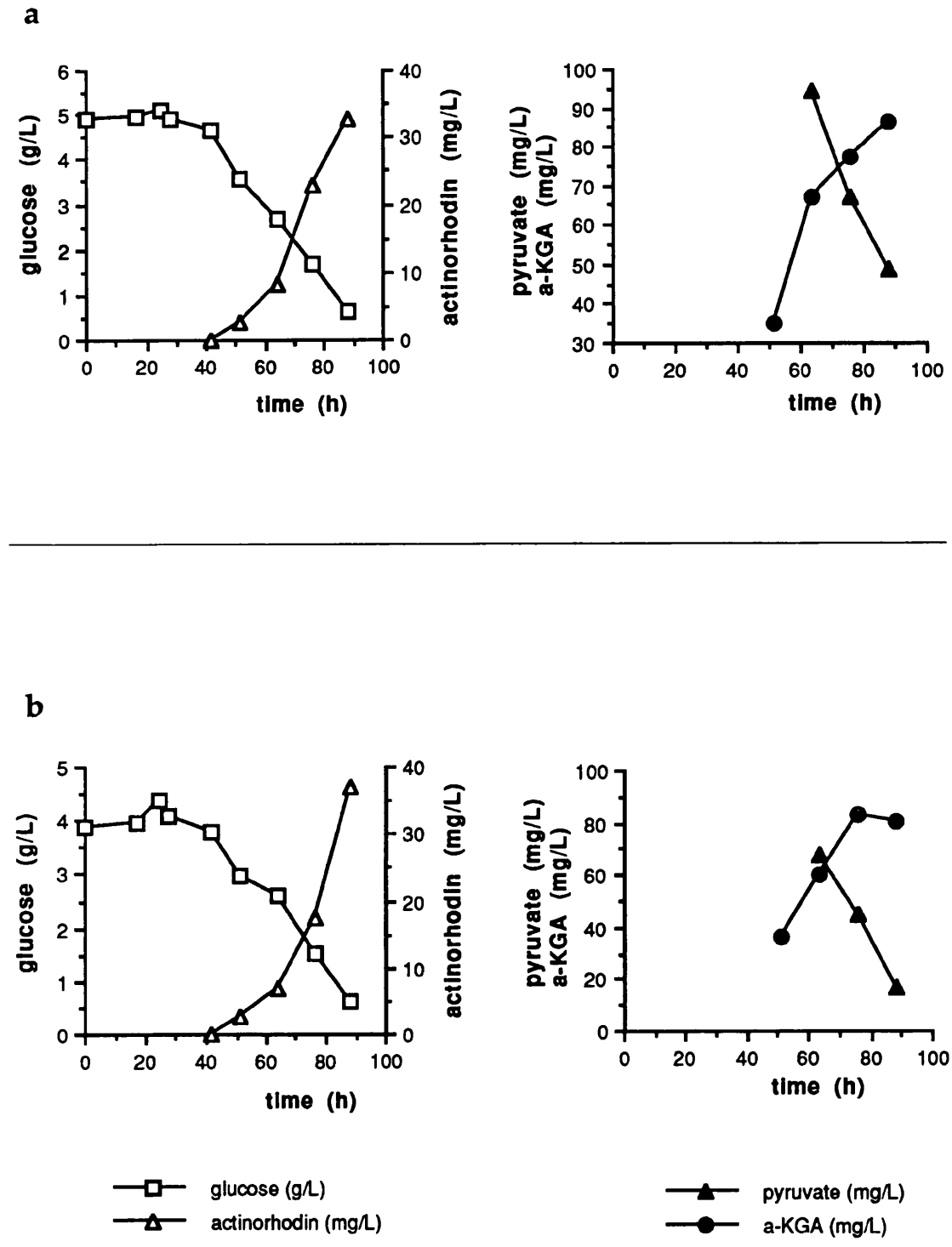



**Figure 5.7. Actinorhodin and organic acid production by *S. coelicolor* 1147 grown in NMM-J in flasks.**
See following page for legend.

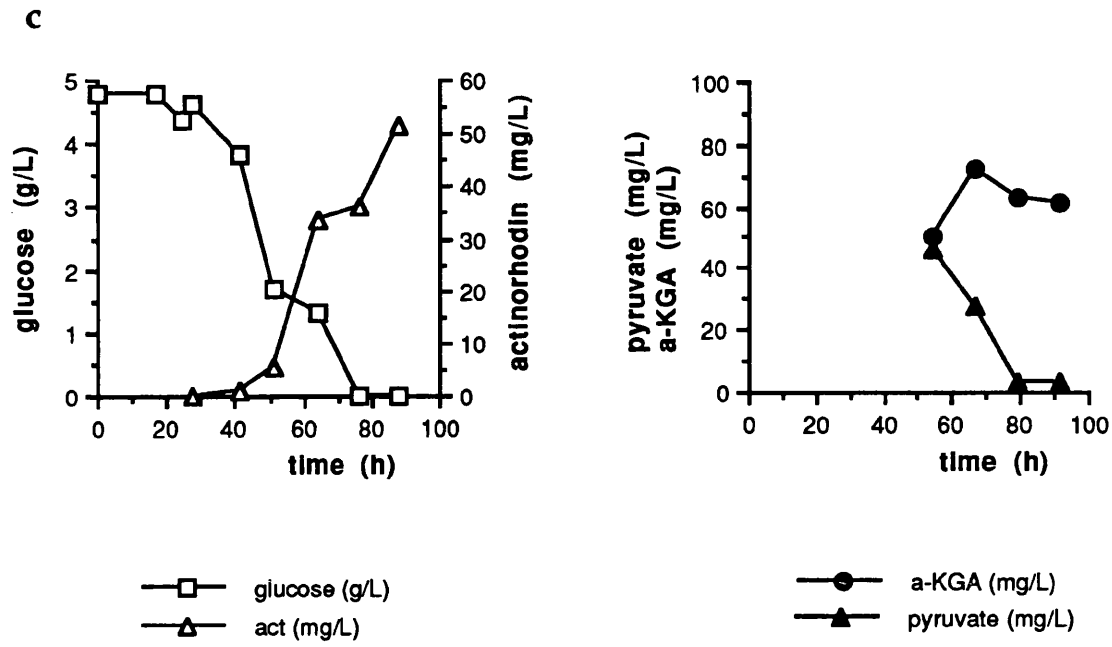



**Figure 5.7** continued.
Triplicate flasks (a, b and c) containing NMM-J were inoculated with *S. coelicolor* 1147 spores and incubated 5 days in an orbital shaker. 1ml samples were removed approximately every 12 hours from the addition of glass beads (at 24h) until actinorhodin was visible in the medium. From then 2ml samples were removed. All samples were assayed for residual glucose (section 2.7.4.1) and actinorhodin (section 2.11). Organic acid production was determined by HPLC (section 2.13.1). act, actinorhodin; α-KGA, α-ketoglutarate.

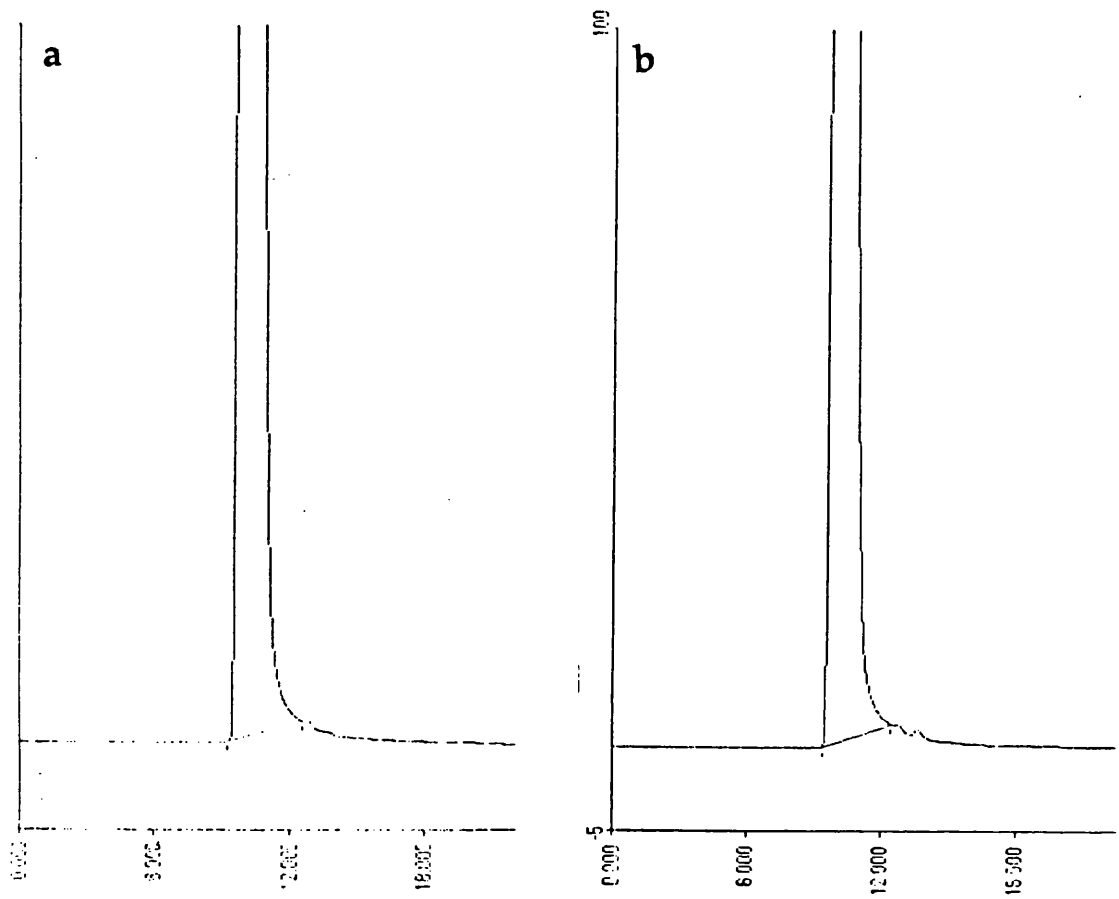



**Figure 5.8. HPLC traces showing time course of production of α-ketoglutarate and pyruvate throughout the cultivation period of *S. coelicolor* in a flask culture.**

Triplicate flask cultures of *S. coelicolor* 1147 were set up as described in figure 5.4. Samples taken at all time points were analyzed for organic acid excretion by HPLC (section 2.13.1). HPLC peaks were identified using authentic standards. The two peaks of interest are to the right of the large peak caused by the anionic compounds in NMM-J; the peak corresponding to α-ketoglutarate is immediately to the right, followed by pyruvate.

**a** 17 hours; **b** 24.75 hours; **c** 27.75 hours; **d** 41.15 hours; **e** 51.4 hours; **f** 63.75 hours; **g** 76 hours; **h** 88 hours.

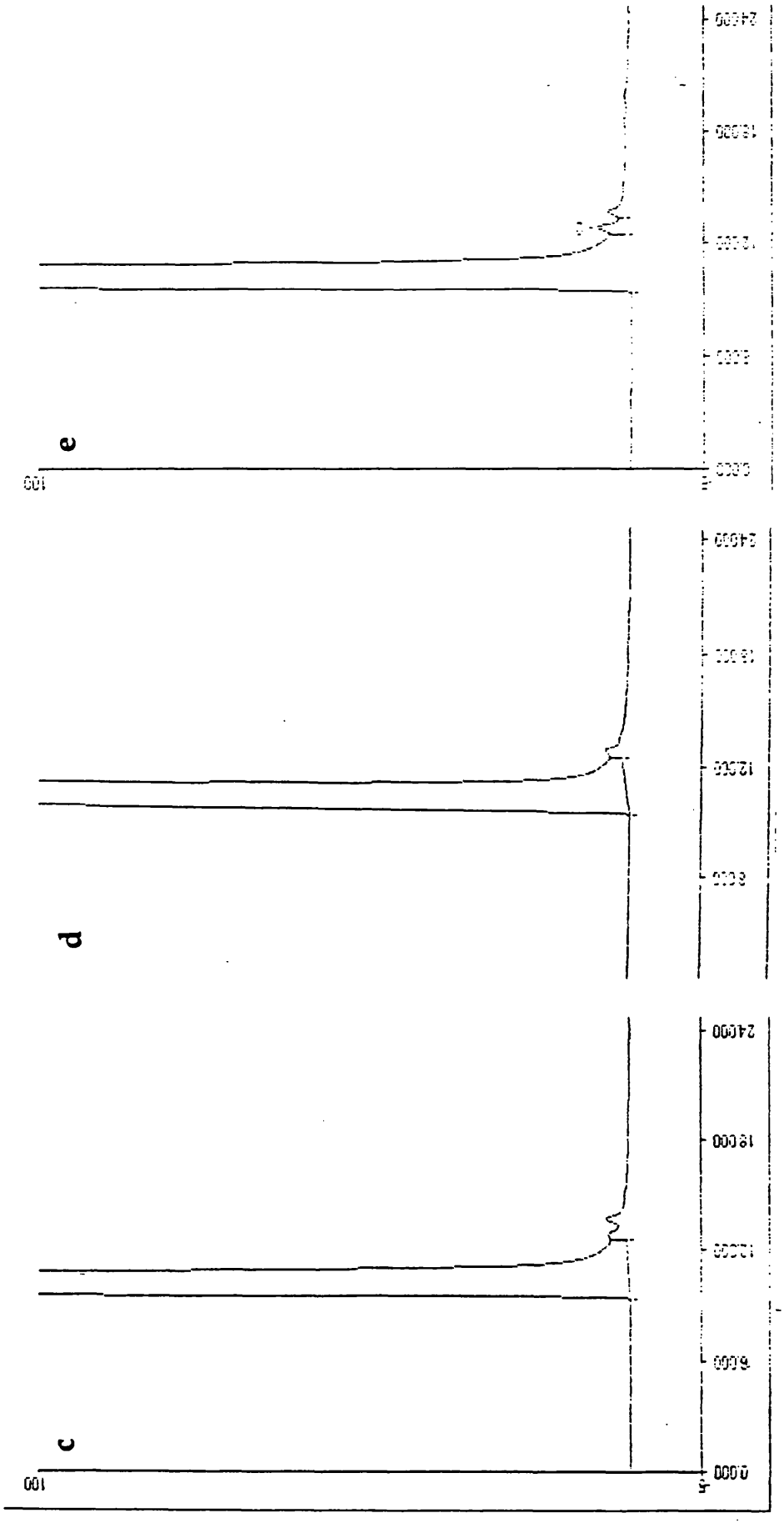



**Figure 5.8 continued.**

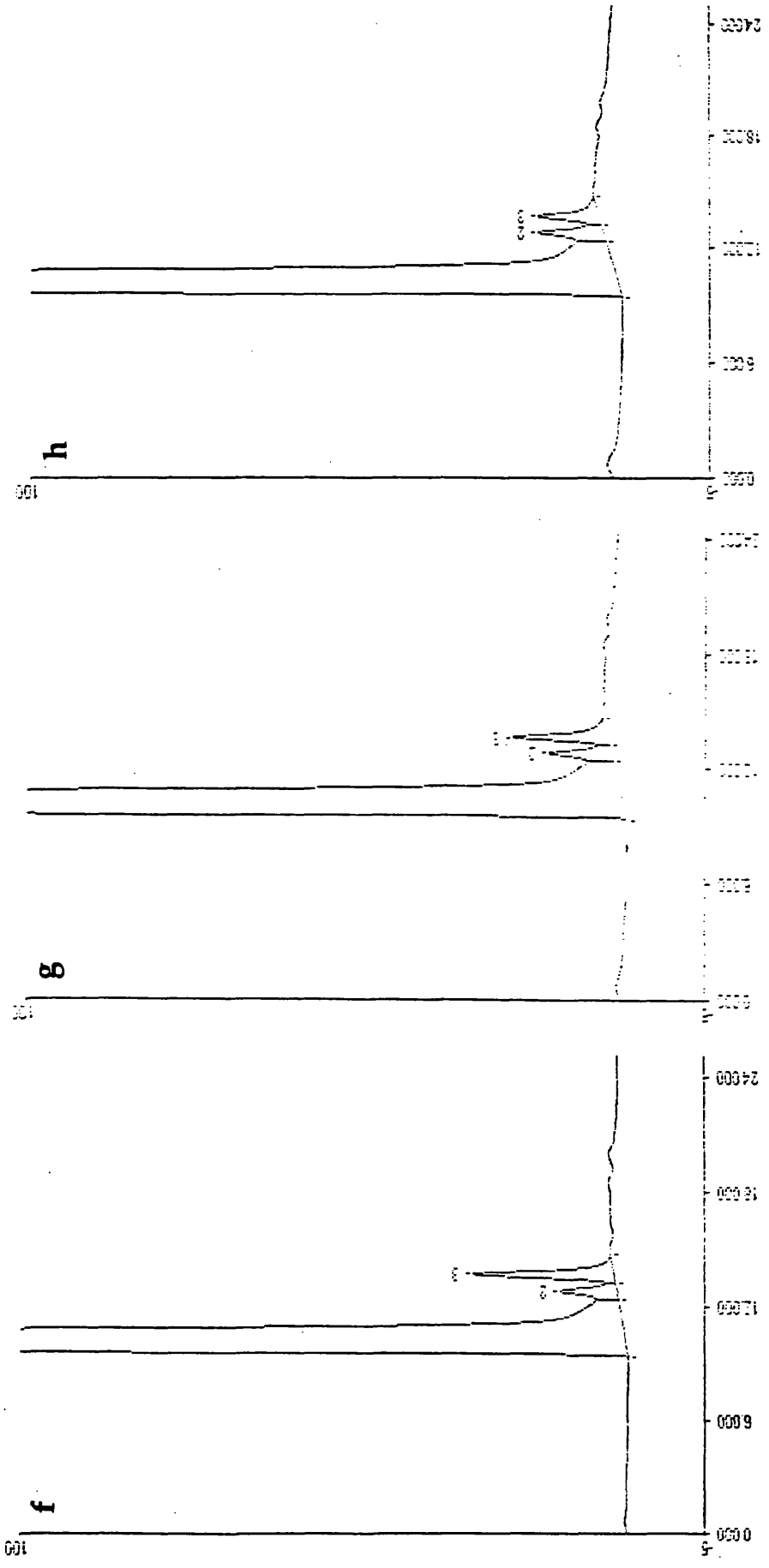



**Figure 5.8 continued.**

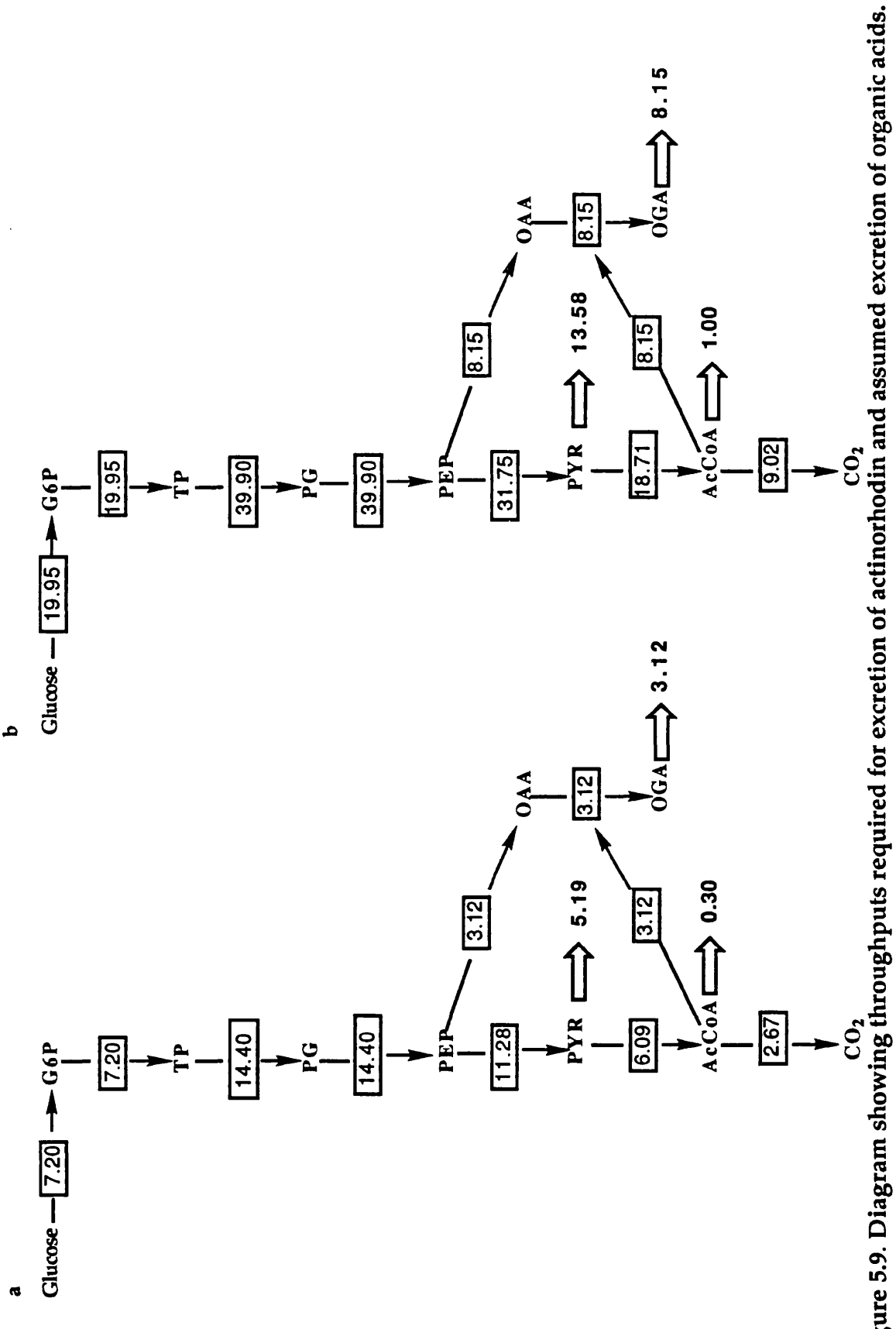



**Figure 5.9. Diagram showing throughputs required for excretion of actinorhodin and assumed excretion of organic acids.**

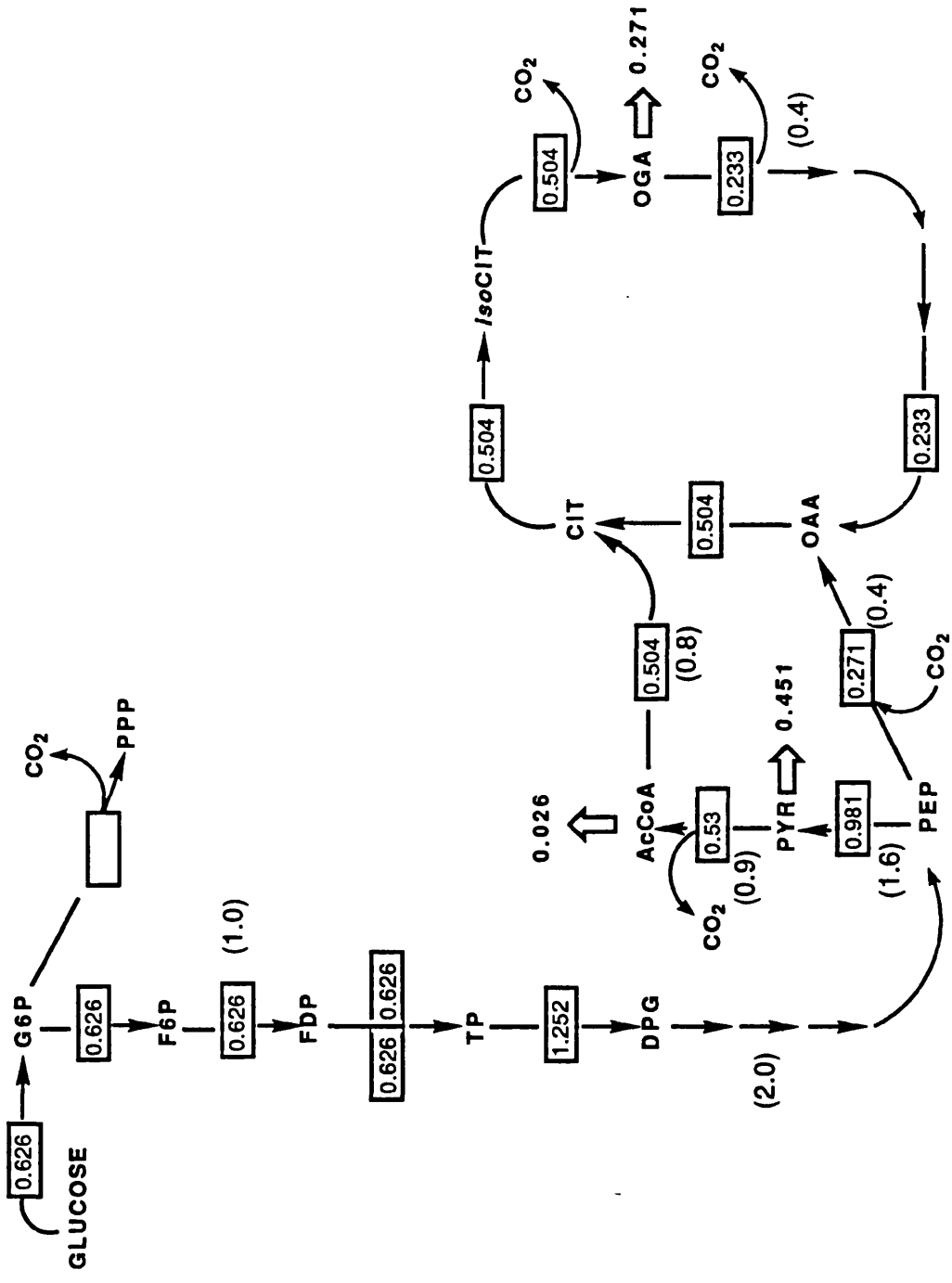



**Figure 5.10a. Fluxes through the CMPs of *S. coelicolor* during production of actinorhodin and assumed excretion of organic acids.** Fermentation C. Figures are in mmol.g$^{-1}$.h$^{-1}$. Values in brackets are ratios of fluxes with respect to flux through PFK.

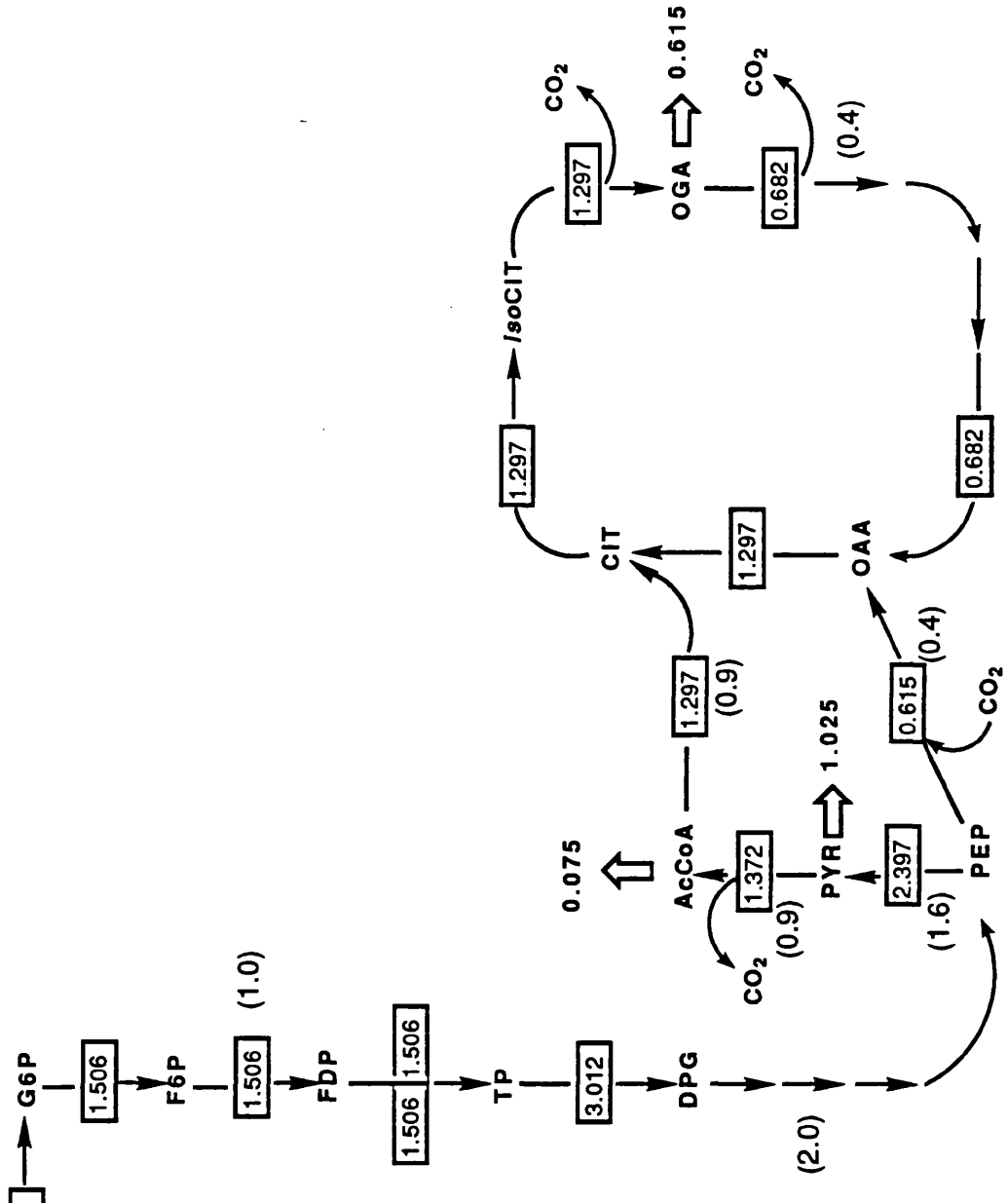



**Figure 5.10** continued. **b** Fermentation D.

**Table 5.4. Ratios of fluxes through assumed enzymes of the central metabolic pathways of *S. coelicolor* to biomass and to excreted metabolite production.**

| | biomass | | excreted products | |
|---|---|---|---|---|
| enzyme | C | D | C | D |
| phosphotransferase-system | 1.1 | 1.1 | 1.0 | 1.0 |
| phosphofructokinase | 1.0 | 1.0 | 1.0 | 1.0 |
| phosphoglycerate kinase | 2.0 | 2.0 | 2.0 | 2.0 |
| enolase | 1.9 | 1.9 | 2.0 | 2.0 |
| pyruvate kinase | 1.8 | 1.8 | 1.6 | 1.6 |
| pyruvate dehydrogenase | 1.6 | 1.6 | 0.8 | 0.9 |
| citrate synthase | 1.5 | 1.5 | 0.8 | 0.9 |
| PEP carboxylase | 0.12 | 0.14 | 0.4 | 0.4 |
| α-ketoglutarate dehydrogenase | 1.4 | 1.4 | 0.4 | 0.4 |

Fluxes to biomass and to excreted metabolite production during secondary metabolism were computed using information from two *S. coelicolor* fermentations and an experiment involving triplicate flasks. Fluxes to biomass (section 5.3.2) were calculated from throughputs obtained from the monomeric composition of *S. coelicolor* (section 4.7). Fluxes to products excreted during secondary metabolism (such as actinorhodin, pyruvate and α-ketoglutarate; section 5.4.2) were calculated, via throughput values, assuming that organic acid excretion observed in triplicate flask cultures had also occurred in the fermentations. Ratios of fluxes were obtained by expressing each flux through the central pathway enzymes (assuming the enzymes were the same as those in *E. coli*) in terms of the smallest flux (excepting that through PEPC), *i.e.*, the flux through phosphofructokinase (PFK).

# Chapter 6

# General Discussion

## 6.1 Introduction

Interest in secondary metabolite production by Actinomycetes, and especially *Streptomyces*, was initiated in the 1940's with the isolation of the antibacterial compounds, actinomycin and streptomycin (Waksman and Woodruff, 1940; Schatz *et al.*, 1944). Since then over 70% of all known natural antibiotics have been isolated from streptomycete species (Berdy, 1980). Interest has also been focussed on the complex life cycle exhibited by *Streptomyces*, where differentiation is closely related to formation of secondary metabolites. Use of molecular genetic techniques has allowed rapid accumulation of knowledge of both antibiotic production and differentiation. However, complementary physiological studies, which are necessary for a better understanding of the streptomycete life cycle, have been hindered by the mycelial nature of *Streptomyces*. This results in the formation of pellets in liquid culture which give rise to cell populations which are heterogeneous.

An important area of study is the switch from primary metabolism into secondary metabolism. During this switch, metabolic fluxes from primary metabolism are diverted to secondary metabolite production. Antibiotics, and other secondary metabolites, are synthesized from intermediates or final products of primary metabolic pathways. Therefore, as a consequence of the switch to secondary metabolism, fluxes to these intermediates, or products, are likely to be altered and possibly increased. Study of the regulation of these "primary" fluxes to secondary metabolite production and subsequent deregulation could result in increased productivity by the organism. Present studies include the purification (and cloning of the genes) of enzymes possibly involved in antibiotic biosynthesis and, therefore, in regulation of the fluxes. These include enzymes of anaplerotic pathways and aromatic biosynthetic pathways. Complementary to this would be the identification of areas in *Streptomyces* metabolism which are actually involved in regulation of the fluxes to antibiotic production. This could be achieved by measurement of the fluxes themselves.

This thesis deals with quantitative measurement of fluxes during primary and secondary metabolism of *S. coelicolor* A3(2). Several requirements were necessary for this, including growth of *S. coelicolor* in a defined minimal medium without the use of carbon-based dispersing agents and determination of the chemical composition of the bacterium. Several discussion points arose from these studies and they will be elaborated upon in the following sections.

## 6.2 Growth of *S. coelicolor* in NMM-J

For the purpose of physiological studies, *S. coelicolor* 1147 was grown in a defined minimal medium, NMM-J. The medium was based on HMM (Hobbs *et al.*, 1989) but the dispersing agent, junlon, was omitted because it was carbon-containing and because it interfered with the chemistry of the methods used to determine carbohydrate and RNA (section 4.3.3). Glass beads were therefore used as a mechanical means of dispersion. However, the difference in growth of *S. coelicolor* in HMM and NMM-J (section 3.3.1) suggested that NMM-J was possibly not suitable for growth in the absence of junlon. The growth differences are expanded upon in this section, as are possible reasons for the relatively slow growth of *S. coelicolor* in NMM-J.

### 6.2.1 Constraints of NMM-J

An obvious difference between growth of *S. coelicolor* in NMM-J and HMM was the morphology. Without the presence of dispersing agents, large, dense pellets were produced in NMM-J resulting in heterogeneous populations of cells. Heterogeneity was obvious because of visible production of the mycelially-associated pigment, undecylprodigiosin, in central areas of pellets but not in the periphery. The presence of glass beads markedly reduced the size and density of the mycelial pellets. Antibiotic production within the mycelia was also not so prominent. Nevertheless, formation of these smaller pellets could still be conducive to nutrient and oxygen limitations within them. This may explain why actinorhodin was produced while mycelial weight continued to increase (section 3.5.1): cells in the centre of pellets may have produced the antibiotic while peripheral cells were still growing. This was also observed in pelleted cultures of *S. akiyoshiensis* in a defined minimal medium where 5-hydroxy-4-oxonorvaline was produced before biomass accumulation ceased (Glazebrook *et al.*, 1992). In NMM-J, *S. coelicolor* did cease to grow a few hours after the onset of actinorhodin production. This eventual co-ordination of metabolism (*i.e.*, the peripheral cells stopped growing and possibly produced the antibiotic) may have been due to signal molecules (pheromones) excreted by the central cells.

In the presence of junlon in HMM, *S. coelicolor* grew in a very dispersed fashion: individual hyphae were well separated and were exposed to the medium. It is not surprising therefore that biomass and actinorhodin production were high. According to Hobbs *et al.* (1990), a final concentration of approximately 2.6g.l$^{-1}$

biomass was obtained in HMM after complete utilization of $2g.l^{-1}$ glucose, a yield of 234 $g.mol^{-1}$. This is rather high as it results in a carbon conversion of 151.8% (assuming *S. coelicolor* biomass comprises 46.7% carbon: section 4.9) but may have been due to junlon sticking to the mycelia. In this work, the optimum amount of dry weight biomass produced by *S. coelicolor* grown in NMM-J, containing $4g.l^{-1}$ glucose, was approximately $1g.l^{-1}$ (section 3.5.1). In flask cultures, this concentration was usually reached after the onset of actinorhodin production, the time of which (around 48 hours; section 3.5.1) was comparable to the onset of production in HMM (Hobbs *et al.*, 1990). In HMM, the glucose was reported to be completely exhausted by this time, whereas in NMM-J a high proportion of glucose remained. This suggests that during trophophase, growth in NMM-J was very slow and uptake of glucose was limited possibly because of the formation of small pellets.

Dispersed growth of *S. coelicolor* has also been demonstrated in an alternative medium containing 5% starch and glass beads. The resulting maximum biomass concentration was $10g.l^{-1}$ which was attributed to the possible viscosity effects of starch (Doull and Vining, 1989). It is therefore possible that, in this work, production of the small quantities of biomass by *S. coelicolor* in NMM-J was partly due to the restricted dispersal effects of the glass beads in a non-viscous medium.

Final concentrations of actinorhodin produced by *S. coelicolor* in each medium were also different. Hobbs *et al.* (1990) reported actinorhodin concentrations of up to $66mg.l^{-1}$. This resulted in antibiotic yields of $25mg.g^{-1}$ of biomass. In the medium containing starch (Doull and Vining, 1989), *S. coelicolor* produced actinorhodin to a maximum concentration of $150mg.l^{-1}$ culture. However, with a biomass concentration of $10g.l^{-1}$, the actual antibiotic yield was 15 $mg.g^{-1}$ biomass. Replacement of the starch with maltose resulted in actinorhodin yields of approximately $58mg.g^{-1}$ biomass. The mycelial wefts formed in the presence of maltose were similar to those formed in NMM-J in shake flasks in this work, where the yield of antibiotic was $30mg.g^{-1}$ biomass. This suggests that pellet formation supports high production of actinorhodin from low amounts of biomass, presumably because of nutrient concentration gradients within pellets but also possibly because of the effects of stress on the mycelia.

In NMM-J, the small pellets exhibited a slightly "battered" look, caused by the shearing effects of the glass beads, and were therefore subjected to a form of stress. Actinorhodin was produced in NMM-J in the presence of excesses of carbon, phosphate and, presumably, nitrogen sources. This was also the case

when *S. coelicolor* was grown in the starch containing medium (Doull and Vining, 1989; 1990). Stress, possibly not induced by nutrient limitation, has been suggested to occur in colonies of *S. coelicolor* grown on solid medium just prior to the onset of differentiation (Granozzi *et al.*, 1990). Differentiation is associated with antibiotic production. It is therefore possible that a similar stress factor is involved in inititation of secondary metabolism. Such a stress factor may also be related to a decrease in growth rate of the organism.

*S. coelicolor* is the streptomycete which is best characterized genetically (Hopwood *et al.*, 1988) and, therefore, many physiological studies, including growth studies, involve *S. coelicolor*. However, with the exception of the two conditions given previously, *S. coelicolor* forms pellets under most conditions (Glazebrook *et al.*, 1992). Several other streptomycete species also exhibit pelleted growth in submerged culture (Williams *et al.*, 1974; Lawton *et al.*, 1989). A recent study has focussed on *S. akiyoshiensis* which forms pellets in a medium similar to that of Doull and Vining (1989), containing 3% starch, 15mM proline and 15mM phosphate (Glazebrook *et al.*, 1992). In an attempt to reduce the extent of pelleting to the dispersed state observed in complex media, Glazebrook *et al.* (1992) altered the different components in the medium. The most effective medium was initially buffered at pH5.5 and contained a high phosphate concentration (15mM) and starch; smaller pellets were formed in the presence of glucose, but biomass accumulation decreased after 2 days. An increase in production of biomass and actinorhodin (per litre) by *S. coelicolor* was observed in a preliminary experiment where phosphate levels were altered (section 3.6). Since increased phosphate reduced the extent of pelleting in submerged cultures of *S. akiyoshiensis* (Glazebrook *et al.*, 1992), it is possible that this increased production of biomass and actinorhodin by *S. coelicolor* was due to better dispersal. Altering additional components of NMM-J may aid in dispersing *S. coelicolor* mycelia further which, in turn, would result in higher quantities of biomass. However, altering the pH and nitrogen source in NMM-J may have negative effects on actinorhodin production since both components were set in NMM-J to allow production of actinorhodin, and to avoid possible interference during production by other secondary metabolites (*e.g.*, methylenomycin).

Additional components in NMM-J which may have had an effect on both biomass and actinorhodin production are the trace salts. In a review by Weinberg (1989), it was shown that the ranges of concentrations of trace metals, *e.g.*, iron and zinc, which permit maximal growth of a selected number of *Streptomyces* species are much wider than the ranges in which optimal yields of

antibiotics occur. NMM-J contained 53.6μM Fe which was within the concentration range of iron for maximal growth of the species examined by Weinberg (1989). However, of the eight *Streptomyces* species examined (*S. coelicolor* was not included), this concentration was only within the range for optimal antibiotic production of two of these species. In contrast, the concentration of zinc in NMM-J (74.7μM) was not within the range for maximal vegetative growth but was in the range required for 75% to 100% of the maximal yield of antibiotics produced by four out of six streptomycete species. It is possible, therefore, that the concentrations of these two metals in NMM-J could have affected growth and actinorhodin production by *S. coelicolor*. As stated previously, biomass yields in NMM-J were very low (0.25g of biomass per g of glucose). A possible factor which may have been involved in inhibiting biomass production could, therefore, have been the concentration of zinc. The higher biomass yields obtained by Doull and Vining (1989; 1990) were possibly due to the carbon and nitrogen sources used. However, zinc may also have been involved since the concentration in the medium (13.8μM) was within the range for optimal growth (Weinberg, 1989). In an examination of the effects of trace metals on growth and actinorhodin production by *S. coelicolor*, Abbas and Edwards (1990) stated that the concentration of zinc used in their medium was found to enhance growth but cause a decrease in actinorhodin production; a concentration of 1.04mM zinc was sufficient to reduce the optimal amount of actinorhodin produced by 50%. Therefore, it is likely that the low yields of actinorhodin produced by *S. coelicolor* when grown in the medium of Doull and Vining (1989; 1990), as mentioned previously in this section, may not have been attributable to zinc.

### 6.2.2 Metabolic energy requirements: maintenance energy

As described in section 5.6, energy directed towards cellular functions not associated with growth, *i.e.*, maintenance energy, is more significant at low growth rates than at high growth rates. At very low growth rates caused by starvation, the maintenance energy requirement of *B. subtilis* was shown to account for greater than 70% of the utilized energy source (Arbige and Chesbro, 1982). In addition, a high proportion of the carbon source may be used for maintenance energy requirements during fermentations in which secondary metabolites are produced. For example, in the late 1970's, 70% of the glucose carbon in the penicillin fermentation was required for maintenance while 20% and 10% was used for production of biomass and penicillin respectively (Heijnen *et al.*, 1979). It is therefore possible that the low yields of *S. coelicolor*

obtained in NMM-J, in comparison to those produced in HMM (Hobbs *et al.*, 1990), were due to utilization of a large proportion of the glucose for maintenance purposes. The high fluxes to $CO_2$ of up to 70% of the carbon input (section 5.3.2) also suggests that a high maintenance energy requirement was present throughout growth in NMM-J.

One requirement for maintenance energy is to ensure solute gradients are upheld. For example, addition of 0.5M NaCl to a fed batch culture of *E. coli* resulted in an increased maintenance energy value and, therefore, a decrease in biomass yield (Wallace and Holms, 1986). A similar experiment with yeast had also resulted in a higher maintenance energy requirement (Watson, 1970). However, it is possible that these cultures of *E. coli* and yeast were energy-limited, since, at low growth rates, cultures of *Klebsiella aerogenes* exhibited no change in oxygen consumption rate with changing osmolarity (Neijssel and Tempest, 1976). An increase in oxygen consumption rate in higher salt concentrations would have indicated an increase in the maintenance energy requirement if the cultures had been energy-limited, since more glucose would have been oxidized to $CO_2$. NMM-J consists of a high proportion of sodium salts: $NaNO_3$ (52.9mM), NaCl (85.6mM) and $Na_2SO_4$ (35.2mM). whereas the basal medium of Doull and Vining (1989) contains only 17.1μM NaCl. The high proportion of glucose oxidised to $CO_2$ during growth of *S. coelicolor* in NMM-J suggests the cultures were energy-limited and that one possible requirement for maintenance energy was to maintain solute gradients in these relatively high salt concentrations.

It was mentioned in section 5.6 that, at low growth rates, more maintenance energy is required for synthesis of the higher quantities of protein present in slowly growing cells. Guanine tetraphosphate is also present in higher concentrations at low growth rates. A significant amount of maintenance energy was calculated to be required for synthesis of ppGpp at these low growth rates (Stouthamer *et al.*, 1990). As described in section 1.5.1, ppGpp may be the effector of the stringent response. ppGpp is synthesized by two pathways; the *relA*-dependent pathway is the best defined and is active during amino acid starvation in *E. coli* (Cashel and Rudd, 1987). The second is a *relA* independent pathway which may be active under conditions of energy limitation (Stouthamer *et al.*, 1990). Very great emphasis has been placed on the role of ppGpp at very low growth rates (Chesbro, 1988; Chesbro *et al.*, 1990) and the molecule has been shown to be important for balanced growth (Stouthamer *et al.*, 1990). *S. coelicolor* is known to synthesise ppGpp (section 1.7.2). It is therefore possible that at the low growth rates exhibited in NMM-J, a

significant proportion of the proposed maintenance energy was used for synthesis of ppGpp which may itself have been involved in regulation of the growth rate (section 1.7.4).

The proposal mentioned above was tested theoretically using data from Bascaran *et al.* (1991) who measured ppGpp levels in mycelia of *S. clavuligerus* grown in different amino acid nitrogen sources (and, therefore, at different growth rates; Bascaran *et al.*, 1990b). This combined data is presented below:

| N-source | doubling time (h) | ppGpp ($nmol.g^{-1}$) |
|---|---|---|
| glutamate | 6.0 | <3.0 |
| glutamine | 8.5 | <3.0 |
| alanine | 13.0 | 5.4 |
| arginine | 21.5 | 6.5 |
| proline | 34.0 | 12.6 |

The rate of formation of ppGpp (in $\mu mol.g^{-1}.h^{-1}$) at the different growth rates was calculated according to Stouthamer *et al.* (1990). The values were then used to determine the contribution of ppGpp formation to maintenance energy by extrapolating the curve of rate of ppGpp formation against growth rate to zero growth rate. A contribution of approximately 7$\mu$moles $ATP.g^{-1}.h^{-1}$ was determined. In *E. coli*, this contribution is 0.19 to 6.3mmoles $ATP.g^{-1}.h^{-1}$ (Stouthamer *et al.*, 1990) which is 27 to 900 fold greater than that calculated for *S. clavuligerus*. In addition, the reported concentrations of ppGpp measured during the stringent response in *S. coelicolor* (*e.g.*, Strauch *et al.*, 1991; Ochi, 1990) was only similar to the concentration of *E. coli* growing with a doubling time of 100 minutes ($\mu = 0.41h^{-1}$). During the stringent response, the rate of formation of ppGpp by *E. coli* was calculated to be $2.1mmol.g^{-1}.h^{-1}$ which was subsequently estimated at a growth rate of $0.015h^{-1}$ (Stouthamer *et al.*, 1990). Therefore, this suggests that the contribution of ppGpp formation at low growth rates to the maintenance energy demand is unlikely to be as important in *Streptomyces* as it is in *E. coli*.

### 6.2.3 Secondary metabolite production in NMM-J

Production of actinorhodin by *S. coelicolor* grown in NMM-J in both batch and continuous cultures has been discussed previously (section 3.8). It was concluded that the onset of production of actinorhodin may have occurred during the period of unbalanced growth, *i.e.*, during the transient periods

between growth and stationary phases in batch culture and between each steady state in the chemostat. In batch cultures, production of undecylprodigiosin was observed prior to production of actinorhodin in both NMM-J and in HMM (Hobbs *et al.*, 1990). It has been suggested that the red pigment is synthesized towards the end of the growth phase (Hobbs *et al.*, 1990; Feitelson *et al.*, 1985). Synthesis of the two antibiotics appears to be differentially controlled: production of actinorhodin is extremely sensitive to levels of ammonium and phosphate whereas synthesis of undecylprodigiosin is only moderately affected (Hobbs *et al.*, 1990).

In a phosphate-limited version of NMM-J, production of undecylprodigiosin by *S. coelicolor* in the chemostat was simultaneous with production of actinorhodin (section 3.7.5). This simultaneous induction of both biosynthetic pathways suggests that conditions in the chemostat during the transient period between steady states should perhaps not be likened to batch growth of *S. coelicolor* as in section 3.8. However, it is possible that the low levels of phosphate in the chemostat during this period were conducive to "early" production of actinorhodin. This may also have been the result of the changes in growth rate which occurred during the transient period.

During steady-state in the chemostat no production of actinorhodin or undecylprodigiosin was observed. However, a yellow pigment was produced which had also been synthesized simultaneously with the antibiotics (section 3.7.5). It is possible that this yellow pigment may have been undecylnorprodigiosin (section 3.8) which is the penultimate product of the undecylprodigiosin pathway (Feitelson *et al.*, 1985). Assuming "yellow" was undecylnorprodigiosin, simultaneous production of the pigment with the final product of the pathway would suggest that a limitation had occurred during the final step of the pathway which is catalysed by undecylnorprodigiosin: S-adenosylmethionine *O*-methyltransferase. This final step was completely prevented during steady-state conditions. Although "yellow" was produced at all steady-states tested, the intensity of the colour decreased with increasing dilution rates which is similar to the observations mentioned by Hobbs *et al.* (1990) concerning production of undecylprodigiosin in the chemostat. It is possible that steady-state conditions in the chemostat did not allow production of undecylprodigiosin in NMM-J at the growth rates used. Alternatively, this suggests that undecylnorprodigiosin: S-adenosylmethionine *O*-methyltransferase may only be active when the metabolism of the mycelia is in an unbalanced state, as in the transient periods between each steady state.

*Streptomyces* have several different σ factors to allow differential transcription of genes (section 1.7.2). Regulation of antibiotic production has been found to be complex (section 1.7.2) and two genes in the *act* pathway have recently been shown to have different promoters corresponding to different σ factors (Parro *et al.*, 1991). It is therefore possible that the gene encoding undecylnorprodigiosin: S-adenosylmethionine *O*-methyltransferase has an alternative promoter to other genes in the *red* biosynthetic pathway. The gene may thus be transcribed by an alternative σ factor which is possibly sensitive to the metabolic state (balanced or unbalanced) of the mycelia. The conditions in the chemostat at the low dilution rates may have been similar to those in the low growth rates experienced by the mycelia at the end of growth phase in batch culture. This may explain why a component of the *red* biosynthetic pathway was produced. However, in the chemostat, the steady-state conditions may not have been suitable for expression of the necessary σ factor.

Alternatively, differential expression of σ factors may be a response to metabolic imbalances or perhaps stress encountered by the mycelia such as those experienced at the end of growth phase in batch culture. Since undecylprodigiosin was only produced in the chemostat during the transient phase, metabolic imbalances under these conditions may have had more influence on antibiotic production than slow growth rates. This could be tested by transferring mycelia producing undecylnorprodigiosin into conditions where the mycelia could reach stationary phase. If the activity of the potential σ factor was associated with metabolic imbalances then the mycelia would produce undecylprodigiosin immediately. This could not be carried out with the biomass from the chemostat, however, because of lysis in the material shipped from Jena (section 3.7.6).

The yellow pigment was also observed during steady-state conditions in a nitrogen-limited version of NMM-J (section 3.7.5). Undecylprodigiosin biosynthesis requires three amino acids (section 1.6.5). It is therefore surprising that, assuming "yellow" was undecylnorprodigiosin, *S. coelicolor* should excrete a nitrogen containing compound during nitrogen limitation. Actinorhodin was not produced at any time during growth in the nitrogen-limited medium but undecylprodigiosin was again produced during the transient period. Production of undecylprodigiosin has been shown to be enhanced at levels of ammonium concentrations below 20mM (and also at similar levels of nitrate; Hobbs *et al.*, 1990). Increased intensities in colour of both the red and yellow pigments were observed in the nitrogen-limited medium as compared with the phosphate-limited medium. Therefore, it is possible that, at the low growth

rates used, it was advantageous for *S. coelicolor* to produce this yellow pigment, albeit possibly nitrogen-containing; S. rimosus has been shown to produce oxytetracycline, which is carbon- and nitrogen-containing, in both carbon- and nitrogen-limited conditions (Rhodes, 1984). Toxicity of some antibiotics, *e.g.*, the aminoglycosides are controlled by covalent modification, usually involving phosphorylation and dephosphorylation (section 1.7.1). Perhaps, if the pigment was undecylnorprodigiosin, possible antibiotic-activation by methylation could have occurred in conditions of metabolic imbalances, as found during the transient phase between steady states. The presence of excess glucose in the steady states may have inhibited the *O*-methyltransferase (or repressed the gene), thus preventing addition of the methyl group.

An intermediate in the biosynthetic pathway of actinorhodin is also yellow (Simpson , 1991) so it is possible that "yellow" is the actinorhodin precursor. The *actI* and *actIII* genes have different promoters, although they are transcribed at similar times and transcription occurs in the presence of glucose (Parro *et al.*, 1991). Therefore, other genes in the *act* pathway may be differentially transcribed leading to excretion of this precursor in the low phosphate concentrations in the chemostat. However, undecylprodigiosin appears to be more growth associated than actinorhodin, and so it is more likely that the yellow pigment could be associated with the *red* pathway.

*S. coelicolor* produces two other antibiotics, methylenomycin and calcium-dependent antibiotic (CDA; section 1.7.2). Using nitrate as a nitrogen source in NMM-J repressed methylenomycin production (Hobbs *et al.*, 1992). However, no examination has been made of the conditions under which CDA is produced. CDA requires calcium for activity (Lakey *et al.*, 1983; Wright and Hopwood, 1983). In the phosphate- and nitrogen-limited modifications of NMM-J, the concentration of calcium was reduced. It is therefore possible that "yellow" was a precursor of CDA which was produced under conditions of lower calcium concentrations. An alternative suggestion is that "yellow" was possibly not related to any of the known antibiotics produced by *S. coelicolor*. This implies that *S. coelicolor* produced a possible novel compound under the conditions imposed by the nutrient limitations in the chemostat.

## 6.3 Chemical composition of *S. coelicolor* grown in NMM-J

Compositional analyses of *S. coelicolor* grown in NMM-J were made at three levels: the polymeric (macromolecular), the monomeric and the elemental

levels. Macromolecular and monomeric data were used to compute fluxes through the central metabolic pathways of *S. coelicolor* but the information obtained at all three levels is useful in its own right. Discussion points concerning the compositions have been raised previously (section 4.9), some of which will be expanded upon in the following sections.

### 6.3.1 Suitability of comparison of compositions of *S. coelicolor* with the composition of *E. coli*

The organism used to demonstrate the determination of fluxes by Holms (1986) was *E. coli*. As described previously (sections 1.3, 1.4), the *E. coli* cell is regarded as a "typical" cell for both genetic and physiological studies. Consequently, more information is available about this bacterium than any other prokaryote. The compositional data of *S. coelicolor* given in this thesis were compared with that of *E. coli* grown in glucose-limited minimal media. Monomeric compositional data on *E. coli* are only available on cells grown at a doubling time of 40 minutes. Consequently, this put limitations on the comparison of a filamentous, streptomycete species with a unicellular enterobacterium. For example, growth of the organisms in glucose minimal media takes place at very different rates. *E. coli* grows with a 40 minute doubling time ($\mu$=1.03$h^{-1}$) whereas the maximum growth rate achieved for *S. coelicolor* in NMM-J was 0.12$h^{-1}$, almost a magnitude less. The macromolecular composition of an organism is known to change with growth rate (Schaechter *et al.*, 1958: Bremer and Dennis, 1987). It is therefore not feasible to compare the macromolecular composition of *S. coelicolor* with that of *E. coli* grown in a glucose minimal medium. However, it may be possible to compare the streptomycete macromolecular composition with the macromolecular composition of *E. coli* grown at a slower growth rate.

At a growth rate of approximately 0.42h ( a doubling time of 100 minutes), *E. coli* was shown to have the composition: 72% protein, 14% RNA, 5% DNA and 9% other components. A high relative proportion of protein is usually observed at low growth rates (Bremer and Dennis, 1987). The relative amount of DNA is also greater because the individual cells are smaller. Consequently, there are more cells and more chromosomes per unit weight. The proportions of RNA and DNA measured in *S. coelicolor* (14% and 6% respectively; section 4.7) were comparable to those in *E. coli* at this growth rate (both compositions were determined by similar colorimetric assays; Bremer and Dennis, 1987: section 2.10). The protein content is much higher in *E. coli* (72%) than in *S. coelicolor*

(53%) but this is probably due to the relative proportions of "other molecules" in the biomass. According to Bremer and Dennis (1987), "other components" comprised 9% of the total composition of *E. coli* at this slow growth rate as compared to 25% at a doubling time of 40 minutes. These components included lipid, lipopolysaccharide, peptidoglycan and glycogen (Ingraham *et al.*, 1983). However, *E. coli* is Gram-negative and therefore differs from Gram-positive *Streptomyces* in content of molecules such as peptidoglycan. *S. coelicolor* has also been shown to contain storage compounds, *e.g.*, polyphosphates (Gray *et al.*, 1990b), and glycogen is suggested to be a possible candidate for the storage compound used in the model of aerial hyphae formation (section 1.2.2; Chater, 1989). Therefore, it is possible that these "other components" comprise a higher proportion of *S. coelicolor* biomass than they do in *E. coli*, and thus reduce the proportion of protein present in the composition of *S. coelicolor*.

An additional difference between *E. coli* and *S. coelicolor* is the method of DNA segregation and division. *E. coli* grows by binary division and the daughter cells obtain equivalent amounts of chromosomal DNA. However, in *Streptomyces*, growth occurs by hyphal elongation at hyphal tips and branch formation (section 1.2.2); daughter cells do not separate and are not equivalent in length, shape and possession of an elongation site (Kretschmer, 1982). In addition, the DNA molecules are segregated heterogeneously. This has been shown by staining nucleoids in *Streptomyces* mycelia (Gray *et al.*, 1990b; Kretschmer, 1987; Kretschmer and Kummer, 1987) where branches were observed to contain much less DNA than the subapical regions of the main hyphae. According to Kretschmer and Kummer (1987), DNA synthesis was not restricted to the apical regions of the hyphae of *S. granaticolor* but also occurred in subapical regions from where it was distributed into developing branches. DNA segregation was equal at the apical tips (*i.e.*, 50%), however, only about 9% of the DNA from subapical cells was distributed into branches. Therefore, although the DNA contents of *S. coelicolor* and *E. coli* grown at a low growth rate may have been similar, this was not reflective of the DNA content of individual cells because the distribution of chromosomes in the two species are very different.

The determined monomeric composition of *S. coelicolor* was also compared to that of *E. coli* as given by Neidhardt (1987). In some respects the compositions were very similar. The nucleotide components were calculated in similar ways for both sets of data. However, some differences may have occurred because of the analytical methods used to determine the two compositions. The amino acid composition of *S. coelicolor* was measured by HPLC (section 4.6.2) but the

method used to determine the amino acid composition of *E. coli* was not stated. Nevertheless, the amino acid composition given by Neidhardt (1987) was very similar to that obtained by Roberts *et al.* (1955) who used radioactive precursors in combination with chromatography. Therefore, it is possible that the method of determination of amino acid composition would not affect the data to a great extent and it was feasible to compare the amino acid composition of *S. coelicolor* with that of *E. coli.* Although *Streptomyces* contain different amounts of some amino acids to *E. coli, e.g.,* a large proportion of glycine in the cell wall (section 4.6.2), it is possible that the majority of amino acids would be present in similar relative proportions. In general, the amino acid contents of the common proteins in the two species could be similar. However, *Streptomyces* DNA has a higher GC content than *E. coli* DNA which may affect the amino acid content of the proteins (section 6.3.2).

It is therefore difficult to say if information about *S. coelicolor* should be compared with *E. coli.* However, more data is available for *E. coli* than any other prokaryote and until more knowledge is available on Gram-positive bacteria and especially about *Sreptomyces, E. coli* may be the only appropriate organism to use for such comparisons.

### 6.3.2 Relationship of *S. coelicolor* amino acid content with the DNA GC bias

The relative amounts of some amino acids in *S. coelicolor* were shown to be very similar to those of the same amino acids in *E. coli* (section 4.6.2). However, certain differences were also evident, the most notable being the amounts of alanine, which was higher in *S. coelicolor,* and methionine, which was lower. It was suggested (section 4.9) that these differences may have been due to the high GC content of the streptomycete DNA. Differences in DNA base composition, especially in GC content, due to mutational pressure on homologous proteins has been recently shown to result in changes in the amino acid composition of the proteins (Muto and Osawa, 1987; Ohma *et al.*, 1989).

The codon usage of 64 streptomycete genes has been determined (Wright and Bibb, 1992). It was shown that there was preferential use in these genes of amino acid codons with G or C in the third position. To allow comparison of the amounts of each amino acid required for translation of 63 of the genes (the highly expressed *EF-Tu* gene of *S. coelicolor* was excluded) with the amino acid composition of *S. coelicolor* derived form this work (section 4.6.2), the percentage proportions of the amino acids determined by Wright and Bibb

(1992) were calculated (table 6.1). The measured amino acid composition was of whole *S. coelicolor* cells, therefore amino acids present in, *e.g.*, the cell wall, were included.

The relative amounts of several of the amino acids were very similar (within 10% error) in both columns in table 6.1, *e.g.*, alanine, methionine, phenylalanine, proline, serine tyrosine and valine. This suggests that the relative amounts of these amino acids present within the 63 genes analysed by Wright and Bibb (1992) were possibly typical for the majority of proteins present within a streptomycete cell. Methionine, phenylalanine and tyrosine are minor components of the composition of *S. coelicolor* and this may be reflective of their restrictive codon sequences. For example, methionine has only one codon (ATG) whereas phenylalanine and tyrosine have two each but they are AT rich (phe: TTT, TTC; tyr: TAT, TAC). Another reason why methionine is present in very low amounts in *S. coelicolor* (as compared to *E. coli*) may be that the ATG initiation codon (for formyl-methionine) is not used in *S. coelicolor* genes to the same extent as in *E. coli* genes. Some streptomycete ORF's have the valine codon GTG as their initiation codon, for example *idh* (encoding isocitrate dehydrogenase) of *S. coelicolor* (Taylor, 1992). Since analysis of the amino-terminal of isocitrate dehydrogenase did not reveal the identity of the initial residue (N-terminal sequence analysis of phosphoglycerate mutase from *S. coelicolor* also did not identify the initial residue; White *et al.*, 1992), it was proposed that the residue was methionine and that it had been cleaved during post-translational processing. However, it is not known if *Streptomyces* use formyl-methionine as the initiation amino acid (IS Hunter, personal communication). Therefore, it is possible that *Streptomyces* prefer the GC rich initiation codon and use valine in the place of formyl-methionine.

The most used amino acids in the 63 gene products (which were also the most abundant amino acids measured in *S. coelicolor*) namely, alanine, arginine, glycine, leucine and valine, had G or C in the first position of their codons and 100% "wobble" in the third position, *i.e.*, G, C, A or T could be used. The amino acid required in the highest quantities was alanine which supported the HPLC measurements on *S. coelicolor* (section 4.6.2). Alanine codons have GC in the initial positions which may explain the presence of the residue as a higher proportion of the amino acid content of *S. coelicolor* as compared to the *E. coli* content. *E. coli* DNA contains equivalent amounts of each deoxyribnucleotide and, therefore, GC rich codons are not abundant relative to AT rich codons.

Observed differences in the use and measured abundancy (section 4.6.2) of the streptomycete amino acids may be due to the limited number of genes available

from *Streptomyces* for determination of their codon usage. It is possible that, in other proteins, amino acids with similar properties, perhaps not solely structural, may be interchanged. For example, positively charged lysine may be replaced by similarly charged arginine (PJ White personal communication; also cited in White *et al.*, 1992) which has some GC rich codons. In addition, differences may be due to the measurement of *S. coelicolor* amino acids from all cellular compartments. *Streptomyces* contain a large proportion of glycine in their cell walls (section 4.6.2) which was indicated by the high glycine content in the amino acid composition of *S. coelicolor*. This would not have been reflected in the codon usage of the genes analysed by Wright and Bibb(1992).

## 6.4 Significance of organic acid excretion by *S. coelicolor* during secondary metabolism

Compositional analyses carried out on *S. coelicolor* grown in NMM-J were used to determine the metabolic fluxes to biomass formation. These fluxes were then compared with the fluxes through the central metabolic pathways during actinorhodin production.

Analytical measurements of metabolites produced by *S. coelicolor* during the period of actinorhodin synthesis in triplicate flask cultures revealed the excretion of two organic acids, pyruate and α-ketoglutarate (section 5.4.1). These organic acids had also been excreted during growth of *S. coelicolor* in NMM-J (but at amounts that were too low to determine). Although these organic acids were measured in three individual flask cultures, it would still be possible that their production was coincident and did not reflect a true correlation with production of actinorhodin. Therefore, it would not be possible to extrapolate this finding to other cultures of *S. coelicolor* grown in NMM-J. However, the following discussion is given in relation to these three cultures.

Hobbs *et al.* (1992) reported the excretion of α-ketoglutarate and pyruvate simultaneously with methylenomycin production by *S. coelicolor* in a modified version of HMM. Organic acid excretion has also been previously shown to occur in cultures of several other streptomycete species (section 1.8.2). Pyruvate and α-ketoglutarate production by *S. venezuelae* coincided with possible nitrogen limitation in a glucose-containing medium, although not all the nitrate had been utilised (Ahmed *et al.*, 1984; section 1.8.2). It was shown that α-ketoglutarate dehydrogenase activity decreased with increasing age of the mycelia and this also supposedly occurred with pyruvate dehydrogenase since

no enzyme activity was detected at the time of glucose exhaustion. No mention of chloramphenicol synthesis by *S. venezuelae* was made by Ahmed *et al.* (1984), therefore it is not known if secondary metabolite production was coincident with organic acid excretion. It was assumed that the nitrogen source in NMM-J ($NaNO_3$) was in excess (A. Moran, personal communication). However, the reducing concentration of nitrate may have been sufficient to affect central metabolism such that organic acid accumulation occurred. It is therefore possible that organic acid excretion by *S. coelicolor* in the limited number of NMM-J cultures measured occurred simultaneously with actinorhodin because of a decrease in activity of the dehydrogenases in similarly aging mycelia. Actinorhodin was produced late in cultivations, so it was feasible that antibiotic production and organic acid excretion would occur at the same time.

The pyruvate dehydrogenase enzyme complex of *E. coli* consists of three components one of which, lipoamide dehydrogenase (E3), is also a component of the α-ketoglutarate complex (Guest, 1974). E3 is encoded by a single gene, *lpd*, which is situated adjacent to the other genes of the pyruvate dehydrogenase complex. However, *lpd* may be expressed independently from its own promoter and a model has been proposed in which transcription of the gene is feedback repressed by free E3 subunits (Nimmo, 1987). In *E. coli*, the genes encoding the other components of the α-ketoglutarate dehydrogenase complex are subject to glucose repression which involves cAMP (Nimmo, 1987). Although cAMP is thought not to have a role in carbon catabolite repression in *Streptomyces* (section 1.), it has been proposed that there may have been suppression of both pyruvate dehydrogenase and α–ketoglutarate dehydrogenase by the carbon source in *S. venezuelae* which resulted in organic acid accumulation (Ahmed *et al.*, 1984).

Since some similarities may exist between regulation of central pathway enzymes in *E. coli* and *Streptomyces*, a model is proposed to explain analytical data from measured cultures for the consecutive excretion of α-ketoglutarate and pyruvate (α-ketoglutarate was observed first) by *S. coelicolor* late in cultivation in NMM-J. It is possible that suppression of α-ketoglutarate dehydrogenase by the glucose in the medium may have occurred as a nutrient source became limiting (perhaps nitrate). Therefore, α-ketoglutarate accumulation would have occurred and E3 would have been present in excess. This would have resulted in feedback repression of *lpd*, which would have affected the level of E3 available for the pyruvate dehydrogenase complex causing subsequent loss of activity. Consequently, pyruvate would also have accumulated.

Alternatively, external influences on *S. coelicolor* in NMM-J may also have affected the fluxes through central metabolism during actinorhodin production. The effect of nitrogen-limitation on the carbon-nitrogen balances has been described previously (section 5.6). However, it is possible that an oxygen limitation may have had some influence. For example, at a dissolved oxygen concentration of 1%, the obligate aerobe *Bacillus stearothermophilus* has been shown to excrete α-ketoglutarate in glucose-sufficient cultures, in addition to acetate and lactate (Martins and Tempest, 1991). The extent of accumulation was higher in $K^+$-limited cultures than in ammonia-limited cultures. Measurement of glycolytic and TCA cycle enzyme activities was carried out during this study by Martins and Tempest (1991). Whereas activities of the glycolytic enzymes increased as the oxygen concentration decreased, activities of some of the TCA cycle enzymes decreased. An oxygen limitation affects the kinetics of dehydrogenases in the TCA cycle because the NAD(P)H produced by them cannot be rapidly oxidised. However, in the *B. stearothermophilus* cultures α-ketoglutarate dehydrogenase activity increased with reducing oxygen concentration although the activity did not match those of the other TCA cycle enzymes, thus α-ketoglutarate was excreted. It is possibe, therefore, that an oxygen limitation which presumably occurred in the small pellets of *S. coelicolor* mycelia in NMM-J may also have accounted for the decrease in the flux through the TCA cycle as actinorhodin was produced. Excretion of pyruvate by *S. coelicolor* may have paralleled the production of lactate by *B. stearothermophilus* because lactate dehydrogenase was present in the Bacillus but not in the streptomycete.

Pyruvate assimilation was also observed in the measured cultures and it is possible that, on solid medium, pyruvate re-utilization may be associated with aerial hyphae formation. On solid medium, differentiation occurs in *Streptomyces* simultaneously with secondary metabolite production. It is thought that the antibiotics produced may have a defensive role at this time (section 1.2.1). Nutrients for growth of aerial hyphae originate from the vegetative mycelia which undergo lysis. It is also possible, however, that excretion of metabolites by the vegetative mycelia may occur prior to lysis; these metabolites may then be utilized by the growing aerial hyphae. Storage of high molecular weight compounds may also occur at this time. Therefore, assimilation of pyruvate could occur for biosynthesis of such compounds. For example, trehalose biosynthesis is known to occur very late during cultivation of *S. parvulus*, possibly during stationary phase (Inbar and Lapidot, 1991). From radioactive labelling patterns, Inbar and Lapidot (1991) proposed that PEP was a precursor in trehalose biosynthesis from exogenous aspartate. It is possible

that pyruvate may be used in a similar fashion to aspartate. Although it is difficult to compare the metabolism of mycelia grown in submerged culture with mycelia grown on solid medium, trehalose is the major carbon source for spore germination and it may be that it is predominantly the aerial hyphae which synthesize trehalose on solid media. Therefore the aerial hyphae could utilize such an excreted molecule for biosynthesis of trehalose.

The determined excretion of α-ketoglutarate and pyruvate was used as an example to show how such metabolite formation would affect fluxes during actinorhodin production by *S. coelicolor*, assuming that these molecules comprised 100% of the carbon excreted into the medium. The flux diagram obtained was used in a comparison of the metabolic fluxes during growth and antibiotic production by *S. coelicolor*. This was done to assist in the identification of enzymes which may be involved in regulation of actinorhodin biosynthesis. Although the example showed that the relative fluxes through the TCA cycle were diminished as compared to those to biomass production, there was no significant change in flux after the branch point to actinorhodin (*i.e.*, from acetyl-CoA). The flux to actinorhodin was calculated as less than 2% of the total input flux and would have been determined as such whether the carbon excreted into the medium was derived from α-ketoglutarate and pyruvate or any other metabolites, *e.g.*, proteins. However, because this flux was so small it was not possible to identify directly an enzyme involved in the regulation of flux to actinorhodin biosynthesis.

However, the studies carried out at present on the deregulation of fluxes in *S. coelicolor* are concentrating on enzymes which could be involved in the control of flux to antibiotic production, *e.g.*, phospho*enol*pyruvate carboxylase (PEPC), isocitrate dehydrogenase (ICDH), isocitrate lyase (ICL) and the shikimate pathway enzymes (section 1.8.2). All these enzymes are involved in fluxes through the TCA cycle. Citrate synthase is also a key enzyme in regulation of the TCA cycle in *E. coli*. It is a large enzyme and is inhibited by NADH and α-ketoglutarate (Weitzman, 1980). However, it is known that there are differences in the properties of citrate synthase throughout the bacterial population. For example, the citrate synthase of Gram-positive, aerobic prokaryotes is small and is not inhibited by NADH or α-ketoglutarate whereas that of the Gram-negative aerobic prokaryotes is large but is also not inhibited by α-ketoglutarate. Therefore, it is likely that citrate synthase in *S. coelicolor* does not exert a similar degree of control on the TCA cycle as in *E. coli*, especially during secondary metabolism. Other central pathway enzymes in Gram-positive prokaryotes have also been found to be different to those in Gram-negative

species. An example is given by pyruvate dehydrogenase in *Bacillus* (Visser *et al.*, 1982). In addition, the isocitrate dehydrogenase of *S. coelicolor* has already been mentioned to differ from the enzyme in other bacterial species (section 1.8.2). Therefore, this method of identifying possible control enzymes because of their involvement in regulation in other bacterial species is problematic.

Until more is known about *Streptomyces* central metabolism it is difficult to do more than compare what little is known about *Streptomyces* with other prokaryotes, and to base studies on what is generally known about central metabolism in *E. coli*. In this way, it could be proposed that a possible candidate enzyme involved in regulation of fluxes to actinorhodin production is PEPC. This is based on the example used to compare the fluxes in *S. coelicolor* during growth and actinorhodin production and also on the observation that PEPC activity increases with time during growth of streptomycete species (section 5.6). It has been suggested that PEPC may have a role in the provision of metabolic precursors or energy for production of secondary metabolites (Dekleva and Strohl, 1988b). In addition, it was shown that increasing gene dosage of PEPC in *E. coli* resulted in decreased acetate excretion (Okungbowa, 1991; section 1.10). It is therefore possible that reducing PEPC activity in *S. coelicolor* may result in increased acetyl CoA accumulation which could be diverted to actinorhodin biosynthesis. However, the biosynthetic pathway of actinorhodin is not known in biochemical terms, neither are possible regulatory areas in the pathway. In addition acetyl CoA stimulates the activity of PEPC (Nimmo, 1987). Therefore, potential problems are envisaged. Nevertheless, it appears that PEPC is a likely candidate enzyme which may be involved in the regulation of actinorhodin biosynthesis and, therefore, the switch to secondary metabolism.

## 6.5 A comparison of fluxes to secondary metabolite production in an "academic" *Streptomyces* species with an "industrial' species

*S. coelicolor* was chosen for this study because it is the most genetically-well characterised of the *Streptomyces* and because the antibiotics it produces have no industrial importance (section 1.11). However, it is obvious from the flux analyses that the actual flux to actinorhodin is extremely small at less than 2% of the total input flux (section 5.4.2). Therefore, any relative differences in fluxes concerning the precursor to actinorhodin, acetyl-CoA, *i.e.*, pyruvate dehydrogenase and citrate synthase, were insignificant. The final discussion point that can now be raised is the feasibility of using such a low-producing

species for such flux analyses, the analytical measurements of which could be overwhelmed by large errors. For the purpose of discussion, the relative carbon flux to actinorhodin was compared with the flux to oxytetracycline (OTC) synthesis in an industrial production strain of *S. rimosus*. OTC is a polyketide (section 1.6.1) and therefore has acetyl-CoA as a central pathway precursor. The flux to OTC was calculated using information kindly given by IS Hunter (personal communication).

During a 10 day fermentation of an industrial strain of *S. rimosus*, $40g.l^{-1}$ of cells were produced in the initial two days. OTC production then occurred and the final concentration in the following 8 days was $50g.l^{-1}$. 8kg of glucose were used to produce 1kg OTC.

Glucose requirement for production of biomass, assuming that biomass contains 48% carbon (section 4.; Burke, 1991; Bushell and Fryday, 1983) and there was a theoretical carbon conversion from glucose to biomass of 55%:

$40g.l^{-1}$ cells = $18.8g.l^{-1}$ carbon

$18.8g.l^{-1}$ carbon produced from $24.18g.l^{-1}$ carbon (at 55% conversion)

*i.e.*, from $\underline{85.45g.l^{-1}\ \text{glucose}}$.

OTC yield = $50/40 = 1.25g.g^{-1}$ cells

= $\underline{2.76\text{mmoles OTC}.g^{-1}\ \text{cells}}$ (MW = 452)

available glucose:

8kg glucose utilized during production of 1kg OTC

*i.e.*, $50g.l^{-1}$ OTC required $400g.l^{-1}$ glucose

$85.45g.l^{-1}$ glucose used for production of biomass

so, $314.55g.l^{-1}$ glucose available for production of OTC

*i.e.*, $7.86g.g^{-1}$ cells = $\underline{43.67\text{mmoles glucose}.g^{-1}\ \text{cells}}$

To calculate fluxes, the time factor of 8 days (192 hours) was used to give:

0.227 mmoles glucose.$g^{-1}.h^{-1}$ → 0.014 mmoles carbon.$g^{-1}.h^{-1}$ (in OTC)

*i.e.*, 1.362 mmoles C.$g^{-1}.h^{-1}$ → 0.308 mmoles C.$g^{-1}$.h (OTC = 22 carbon atoms)

*i.e.*, a relative flux to OTC of $\underline{22.6\%}$.

In a similar way, assuming a carbon conversion from glucose to biomass of 40%, 25.1% of the input flux was calculated to flow to biosynthesis of OTC in

this fermentation. These carbon fluxes to OTC of 22.6% and 25.1% of the input carbon flux reveals that there is still much scope for improvement using physiological studies of this industrial strain of *S. rimosus*. Nevertheless, the flux to OTC is much greater than the flux to actinorhodin. Flux analyses of *S. rimosus* during OTC production would therefore possibly show definite differences in fluxes through specific central pathway enzymes as compared to those determined during biomass production. Determination of such a relatively large flux to OTC would not be affected by interference from analytical errors, unlike the flux to actinorhodin. *S. rimosus* may also grow better in a minimal medium. Consequently, wild-type *S. coelicolor* and actinorhodin production may not be suitable choices for further flux analysis.

## 6.6 Conclusions and future work

The results presented in this thesis form only a preliminary investigation into the composition of *S. coelicolor* grown in a defined minimal medium and into the fluxes to biomass and antibiotic production through the central metabolic pathways of this streptomycete species. The analyses were hindered because of the limitations on dispersed growth imposed by the medium and by the use of glass beads as shearing agents. Reproducible cultures were not obtained either in flasks or in a 7litre fermenter. However, extremely dispersed growth was observed during continuous cultivation of *S. coelicolor*. Nevertheless, an average macromolecular composition was obtained which was similar to the composition of *E. coli* when grown at a slow growth rate. The determined monomeric composition of *S. coelicolor* was also comparable to that of *E. coli*. In addition, the amino acid content of *S. coelicolor* contained some similarities to the use of amino acids in 63 streptomycete proteins as determined by codon usage analysis.

An attempt was made to use the compositional data of *S. coelicolor* to compare the fluxes through central metabolism during growth and actinorhodin production. However, because the flux to actinorhodin was small it was not useful in the possible identification of an enzyme(s) involved in regulation of the synthesis of the antibiotic. Nevertheless, PEPC was suggested to be a possible candidate enzyme for such a regulatory role.

This study has provided basic groundwork on the quantitative physiology of *S. coelicolor*. However, it is important that if *S. coelicolor* is to be used for further physiological studies more work should be carried out to obtain dispersed

growth in a minimal medium which does not contain a carbon based dispersing agent. Alternatively, a different *Streptomyces* species could be used for such investigations, perhaps a species which grows in a well-dispersed fashion in defined media and which produces a greater yield of secondary metabolites. *S. coelicolor* was chosen for these studies because of the extent of knowledge about the genetics of the species. However, because so much is known about *S. coelicolor* and it is regarded as "typical" with respect to streptomycete genetics, it is possible that it will take less time to discover more about other streptomycete species.

Nevertheless, one suggestion for future work concerning deregulation of the fluxes to actinorhodin biosynthesis in *S. coelicolor* would be to concentrate on the enzyme PEPC. This enzyme has been purified recently and its gene cloned. Therefore, such future work would attempt to modify the activity of the enzyme. Reduction of PEPC activity may result in an increase in flux from acetyl-CoA to actinorhodin. This could be achieved by mutating the gene or its controlling element(s).

**Table 6.1. Comparison of the amino acid composition of *S. coelicolor* with the composition determined from codon usage analysis.**

| amino acid | *S. coelicolor* (mean) % | *Streptomyces* codon usage[a] % |
|---|---|---|
| alanine | 14.1 | 13.1 |
| arginine | 6.5 | 8.1 |
| aspartate} | 6.1 | 6.4 |
| asparagine} | | 2.5 |
| cysteine | nd | 0.8 |
| glutamate} | 7.2 | 5.9 |
| glutamine} | | 2.6 |
| glycine | 14.3 | 9.1 |
| histidine | 1.7 | 2.5 |
| isoleucine | 4.5 | 3.3 |
| leucine | 7.8 | 9.2 |
| lysine | 5.8 | 2.4 |
| methionine | 1.5 | 1.6 |
| phenylalanine | 3.2 | 2.9 |
| proline | 5.8 | 5.5 |
| serine | 4.7 | 5.3 |
| threonine | 5.7 | 6.8 |
| tryptophan | nd | 1.6 |
| tyrosine | 1.9 | 2.3 |
| valine | 8.3 | 7.9 |

The amino acid composition of eight *S. coelicolor* biomass samples harvested from four fermentations (section 4.6.2) were determined by HPLC analysis (section 2.12). The amounts of amino acids per gram dry weight were expressed as a percentage of the total amino acid content (table 4.18). The means of these percentages are presented. The relative use of the codons for each amino acid was calculated from the individual codon useage of 63 streptomycete genes (Wright and Bibb, 1992).

# Appendices

# Appendix A

**Methodology for determination of amino acid composition of *S. coelicolor*.**

The method used for hydrolysis of *S. coelicolor* proteins (section 2.12.2.1) was suitable for the determination of most amino acids. However, asparagine and glutamine were hydrolyzed to aspartate and glutamate respectively during acid hydrolysis and therefore appeared as these peaks. Cysteine and tryptophan were also not detected during these determinations because they required alternative means of determination; the HPLC methods used were under routine use. Cysteine was oxidized to cystine and tryptophan was unstable during acid hydrolysis (Inglis, 1983).

Gradient method 1a (section 2.12.2.5) resulted in good separation of most amino acids from *S. coelicolor* biomass excluding histidine and glycine peaks (figure A1a). Co-elution of these residues had also been observed using standards. This was rectified by using an isocratic method (1b; section 2.12.2.5) in which *o*-phthaladahyde-mercaptoethanol (OPA-mercaptoethanol) replaced OPA-mercaptopropionate. Mercaptoethanol is uncharged, unlike propionate, and therefore caused retention of the amino acids over a longer time period at pH6.0. The first six amino acids (aspartate, glutamate, histidine, glycine, threonine and alanine) were eluted over 20 minutes by this method (figure A1b).

Resulting amino acid concentrations of two biomass samples harvested at different times from the same fermentation were calculated with respect to external standards using the equation below. DF is the dilution factor used.

$$\frac{\text{sample peak area}}{\text{standard peak area}} \times 0.1\text{mM} \times \text{DF} = \text{amino acid concentration}$$

For each biomass sample, hydrolysis was performed for 24, 48 and 72 hours. The amino acid compositions of the hydrolysates were determined independently (section 2.12.2.1). However, no general patterns were revealed which could identify amino acids as stable, unstable or released slowly. In this way, two samples of *S. coelicolor* biomass harvested from the same fermentation were analyzed for amino acid concentration (table A1). Sample 1 was harvested before actinorhodin production while sample 2 was obtained during antibiotic production. Alanine was at the highest concentration observed in both samples,

followed by glycine, leucine and valine. Methionine was present at the lowest concentration. The majority of amino acids in sample 2 were, however, approximately half the concentration of those in sample 1 resulting in the total amino acid content of sample 2 summing to 39.5% dry weight biomass. Hydrolysis of the samples was therefore repeated and the samples were re-analyzed.

During further analysis, resolution of alanine deteriorated in the gradient system. To compensate for this, the isocratic system was lengthened to 35 minutes to allow elution of alanine (method 2b; section 2.11.2.5). The gradient method was reduced to 28 minutes with an initial solvent ratio of 90% solvent A to 10% solvent B (method 2a; section 2.11.2.5). This caused co-elution of the first seven amino acids (figure A2) which were resolved in the isocratic system. Use of this method with the re-hydrolyzed samples resulted in similar amino acid concentrations in both samples 1 and 2. Expression of these concentrations in terms of biomass dry weight gave figures (52.4% and 65.2% respectively; table A2) which agreed with the protein content obtained by carbon analysis of the alkali fractions (section 4.4.2).

Methods 1 and 2 (section 2.12.2.5) had allowed the determination of sixteen amino acids in *S. coelicolor* biomass samples. Additional amino acids were determined in samples from other fermentations by altering the solvent constituents (method 3a; section 2.12.2.5). Method 3c was used for the resolution of proline. The slight change in conditions for the other amino acids resulted in exchange in retention times of methionine and valine peaks and also those of phenylalanine and leucine (figure A3). An unidentified peak observed to elute before alanine was subsequently identified as N-acetylglucosamine (by "spiking" the samples with appropriate standards). An N-acetylglucosamine standard was therefore used separately for quantitation of this peak.

Long term use of method 3a with the HPLC column for analysis of several samples resulted in co-elution of the internal standard, ε-n-amino caproic acid, with phenylalanine. This was resolved by using the isocratic method 3b (section 2.12.2.5).

A final table (table A3) is included in this appendix. Included in the table are the standard deviations from the amino acid compositions described in table 4.17.

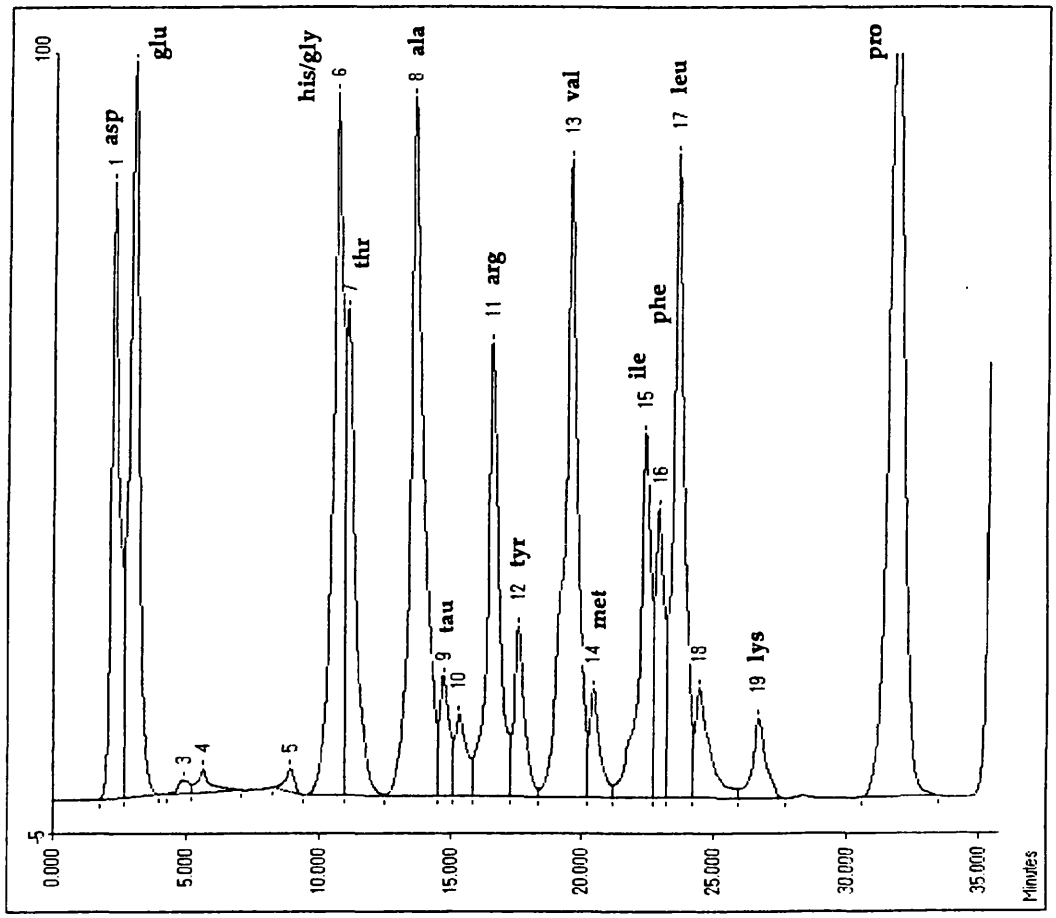



**Figure A1. HPLC trace from amino acid analysis of an *S. coelicolor* biomass sample using analysis method 1.** Biomass samples taken from an *S. coelicolor* culture grown in the Bioengineering fermenter were subjected to acid hydrolysis to liberate the amino acids (section 2.12.2.1). The samples were then analyzed for amino acid content by HPLC using analysis method 1 (section 2.12.2.5). HPLC peaks were identified using a mixture of authentic amino acid standards.

a HPLC trace of an *S. coelicolor* biomass sample harvested at 40hours after inoculation of the fermenter, *i.e.*, prior to the onset of production of actinorhodin. This sample was a 5-fold dilution of the 72 hour hydrolysate of the biomass sample and was analyzed for amino acid content using gradient method 1a (section 2.12.2.5). asp, aspartate; glu, glutamate; his/gly, histidine/glycine; thr, threonine; ala, alanine; tau, taurine (internal standard); arg, arginine; tyr, tyrosine; val, valine; met, methionine; ile, isoleucine; phe, phenylalanine; leu, leucine; lys, lysine; pro, proline.

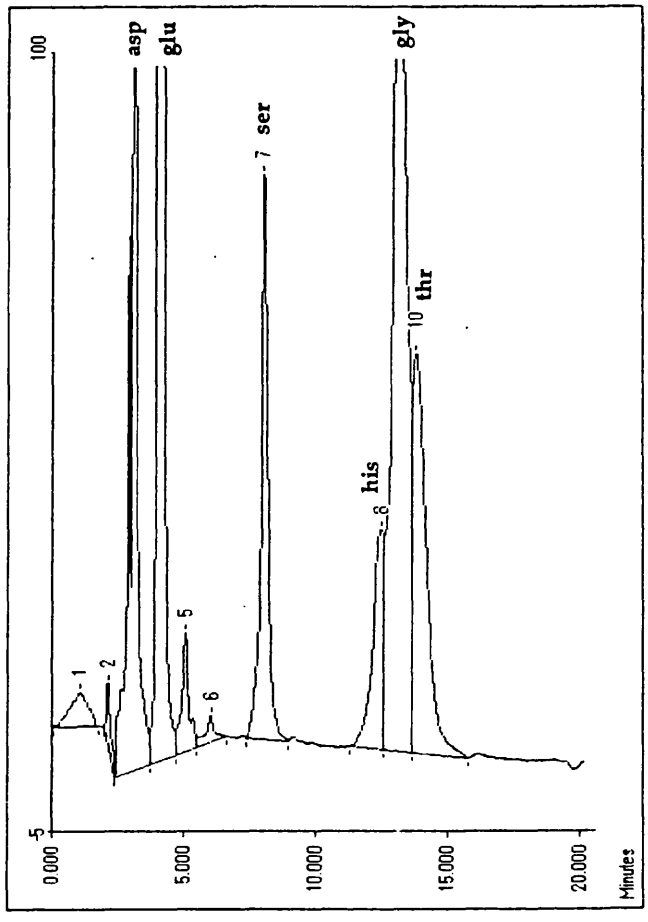



**Figure A1** continued.
**b** HPLC trace of the same hydrolysate described in figure A1a but the method of analysis was isocratic method 1b (section 2.12.2.5). The sample was analyzed in an undiluted form. asp, aspartate; glu, glutamate; ser, serine; his, histidine; gly, glycine; thr, threonine.

**Table A1. Amino acid concentrations of *S. coelicolor* biomass samples.**

| amino acid | sample 1 (mM) | sample 2 (mM) |
|---|---|---|
| asparate | 0.869+/-0.068 | 0.542+/-0.104 |
| glutamate | 0.848+/-0.071 | 0.562+/-+/-0.08 |
| serine | 0.493+/-0.128 | 0.2230.062 |
| histidine | 0.307+/-0.068 | 0.168+/-0.008 |
| glycine | 1.219+/-0.047 | 0.64+/-0.166 |
| threonine | 0.608+/-0.085 | 0.27+/-0.113 |
| alanine | 1.802+/-0.394 | 1.014+/-0.115 |
| arginine | 0.767+/-0.138 | 0.381+/-0.063 |
| tyrosine | 0.245+/-0.039 | 0.114+/-0.033 |
| valine | 0.964+/-0.157 | 0.529+/-0.045 |
| methionine | 0.156+/-0.033 | nd |
| isoleucine | 0.488+/-0.07 | 0.273+/-0.03 |
| phenylalanine | 0.305+/-0.051 | 0.182+/-0.011 |
| leucine | 0.999+/-0.139 | 0.553+/-0.084 |
| lysine | 0.71+/-0.308 | 0.344+/-0.077 |
| proline | 0.622+/-0.06 | 0.363+/-0.034 |

Triplicate aliquots of two samples harvested from the same *S. coelicolor* fermentation were hydrolyzed in 6N HCl for 24, 48 and 72 hours to release protein components (section 2.12.2.1). The samples were analyzed for amino acid concentration by HPLC using solvent method 1 (section 2.12.2.5) based on the elution profile of a mixture of common amino acid standards. Concentrations in the sample aliquots were then expressed as means plus/minus their standard deviations.

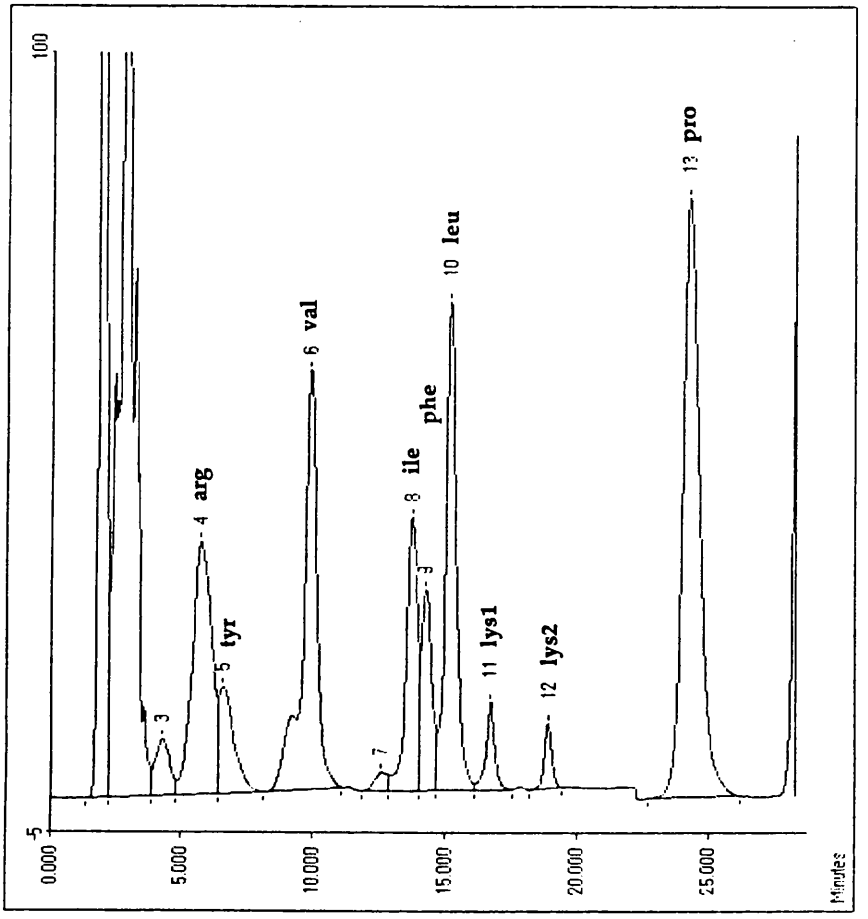



**Figure A2. HPLC trace of an *S. coelicolor* biomass sample analyzed for amino acid content using an alternative gradient method.** A biomass sample was harvested from a culture of *S. coelicolor* grown in the Bioengineering fermenter during the time of production of actinorhodin. The sample underwent acid hydrolysis to liberate the amino acids from the proteins (section 2.12.2.1). The sample shown in the figure is the 48hour hydrolysate of the biomass sample, which was analyzed, at 5-fold dilution, using gradient method 2a (section 2.12.2.5). arg, arginine; tyr, tyrosine; val, valine; ile, isoleucine; phe, phenylalanine; leu, leucine; lys, lysine; pro, proline.

**Table A2. Amino acid concentrations of *S. coelicolor* biomass samples. Method 2.**

| amino acid | sample 1 (mM) | sample 2 (mM) |
|---|---|---|
| aspartate | 0.733+/-0.031 | 0.73+/-0.075 |
| glutamate | 0.803+/-0.064 | 0.813+/-0.11 |
| serine | 0.47+/-0.087 | 0.427+/-0.025 |
| histidine | 0.32+/-0.066 | 0.283+/-0.13 |
| glycine | 1.177+/-0.058 | 1.3+/-0.265 |
| threonine | 0.717+/-0.081 | 0.65+/-0.01 |
| alanine | 1.417+/-0.36 | 1.43+/-0.061 |
| arginine | 0.907+/-0.338 | 0.804+/-0.177 |
| tyrosine | 0.214+/-0.133 | 0.288+/-0.075 |
| valine | 0.946+/-0.154 | 0.828+/-0.031 |
| methionine | 0.02+/-0.013 | <0.005 |
| isoleucine | 0.427+/-0.045 | 0.393+/-0.085 |
| phenylalanine | 0.328+/-0.091 | 0.318+/-0.038 |
| leucine | 0.883+/-0.149 | 0.822+/-0.06 |
| lysine | 0.523+/-0.056 | 0.478+/-0.106 |
| proline | 0.603+/-0.093 | 0.563+/-0.176 |

*S. coelicolor* biomass samples 1 and 2 were again hydrolyzed in 6N HCl (section 2.12.2.1) and the resulting aliquots were analyzed by HPLC using method 2 (section 2.12.2.5).

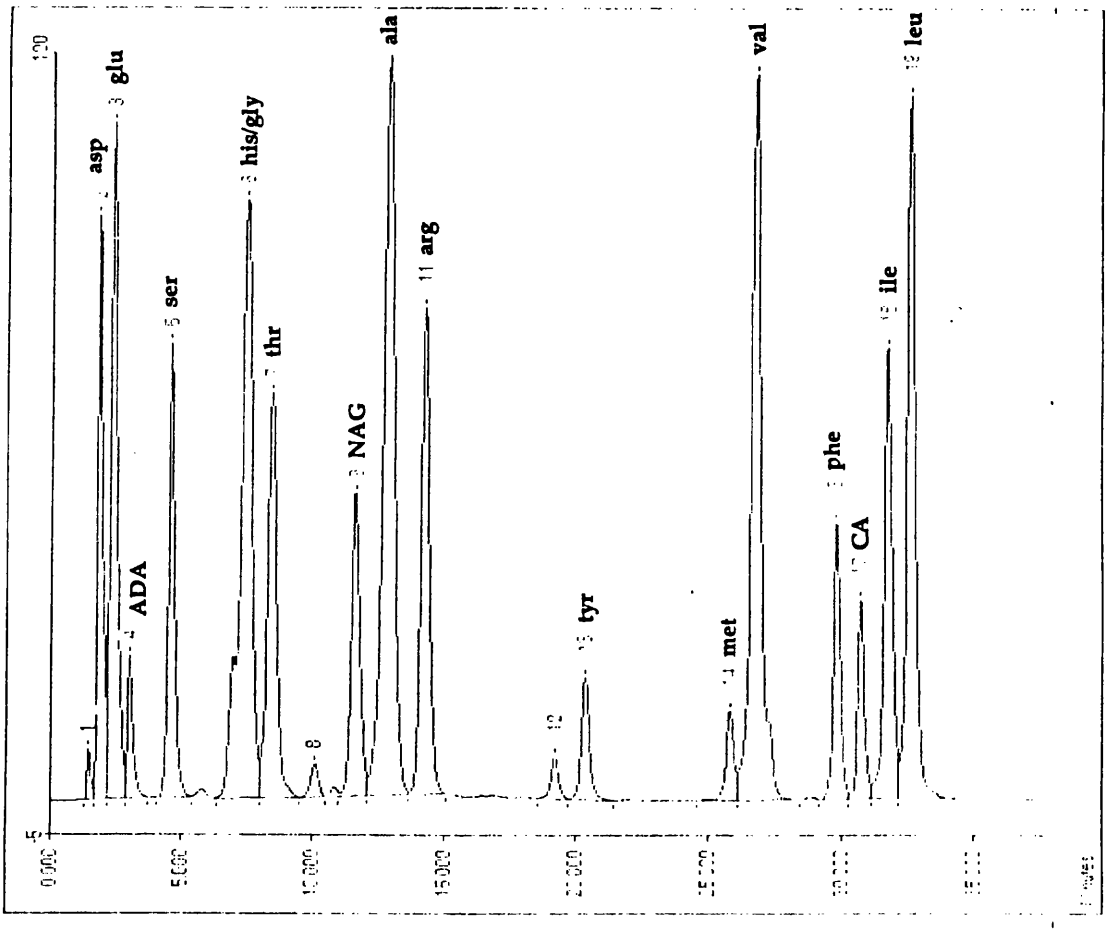



**Figure A3. HPLC trace of *S. coelicolor* biomass sample analyzed for amino acid content by analysis method 3.** A biomass sample was harvested from a culture of *S. coelicolor* 1147 growing in the Bioengineering fermenter. The sample was taken before the mycelia produced actinorhodin. The amino acid content of the sample was analyzed, following acid hydrolysis, by HPLC using gradient method 3a. The sample shown is the 24hour hydrolysate of the biomass sample which was analyzed at a 2-fold dilution. asp, aspartate; glu, glutamate; ADA, α-amioadipate; ser, serine; his/gly, histidine/glycine; thr, threonine; NAG, N-acetylglucosamine; ala, alanine; arg, arginine; tyr, tyrosine; met, methionine; val, valine; phe, phenylalanine; CA, caproic acid (internal standard); ile, isoleucine; leu, leucine.

**Table A3. Standard deviations from means of amino acid compositions described in table 4.17.**

| | SAMPLES | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A | B | C | D | E | F | G | H |
| alanine | 0.009 | 0.061 | 0.054 | 0.15 | 0.007 | 0.055 | 0.041 | 0.041 |
| arginine | 0.005 | 0.033 | 0.054 | 0.141 | 0.002 | 0.034 | 0.035 | 0.011 |
| aspartate | 0.001 | 0.014 | 0.017 | 0.013 | 0.000 | 0.009 | 0.031 | 0.142 |
| cysteine | nd | nd | nd | nd | nd | nd | nd | nd |
| glutamate | 0.005 | 0.013 | 0.022 | 0.027 | 0.000 | 0.018 | 0.032 | 0.129 |
| glycine | 0.033 | 0.066 | 0.086 | 0.024 | 0.024 | 0.082 | 0.061 | 0.083 |
| histidine | 0.004 | 0.011 | 0.018 | 0.028 | 0.004 | 0.040 | 0.012 | 0.016 |
| isoleucine | 0.007 | 0.016 | 0.039 | 0.019 | 0.004 | 0.035 | 0.030 | 0.004 |
| leucine | 0.008 | 0.037 | 0.028 | 0.0.062 | 0.002 | 0.034 | 0.043 | 0.015 |
| lysine | 0.019 | 0.040 | 0.051 | 0.0.023 | 0.007 | 0.035 | 0.054 | 0.034 |
| methionine | 0.0005 | 0.012 | 0.032 | 0.005 | 0.002 | 0.013 | 0.013 | nd |
| phenylalanine | 0.006 | 0.015 | 0.026 | 0.038 | 0.001 | 0.015 | 0.035 | 0.012 |
| proline | 0.018 | 0.39 | 0.035 | 0.039 | 0.005 | 0.034 | 0.041 | 0.018 |
| serine | 0.004 | 0.030 | 0.029 | 0.036 | 0.004 | 0.024 | 0.024 | 0.031 |
| threonine | 0.008 | 0.028 | 0.034 | 0.034 | 0.006 | 0.025 | 0.050 | 0.035 |
| tryptophan | nd | nd | nd | nd | nd | nd | nd | nd |
| tyrosine | 0.002 | 0.010 | 0.020 | 0.055 | 0.000 | 0.008 | 0.008 | 0.013 |
| valine | 0.009 | 0.39 | 0.040 | 0.064 | 0.002 | 0.037 | 0.045 | 0.033 |

# Appendix B

## Determination of nucleotide content of *Streptomyces* RNA.

*E. coli* RNA contains 81.5% rRNA, 14.5% tRNA and 4.0% mRNA. The nucleotide content of RNA has been calculated as 26.2% AMP, 32.2% GMP, 20% CMP and 21.6% UMP (Neidhardt, 1987, based on data from Roberts *et al.*, 1955). This is different to the DNA content of 24.6% dAMP, 25.4% dGMP, 25.4% dCMP and 24.6% dTMP (Neidhardt, 1987) because rRNA is the major species in total RNA (section 4.1) and has a composition different from that of total RNA. To determine the nucleotide content of *Streptomyces* RNA, it was therefore necessary to examine the published sequences of all known *Streptomyces* RNA species.

*Streptomyces* DNA is approximately 70% GC rich and this would be reflected in the GC mol % of mRNA. However, the sequences of other RNA species showed lower GC contents:

**rRNA**

58.5% (Suzuki and Yamada, 1988)
58.9% (Baylis and Bibb, 1987)
average 58.7% GC rich.

**tRNA**

| | |
|---|---|
| $tRNA^{gly}$ | 64% (Rokem *et al.*, 1990) |
| $tRNA^{gln1,2}$ | 51% (Plohl and Gamulin, 1990) |
| $tRNA^{glu1,2,3}$ | 70.3% (Plohl and Gamulin, 1990) |

average 61.8% GC rich.

To determine the amount of each individual nucleotide in *Streptomyces* RNA each base was counted in all the above mentioned sequences. The values were then expressed as a percentage of the total bases in the sequence:

| | A(%) | G(%) | C(%) | U(%) | |
|---|---|---|---|---|---|
| **rRNA** | 22.75 | 32.9 | 25.8 | 18.55 | (mean of 2) |
| **tRNA** | 16.0 | 33.2 | 29.5 | 23.2 | (mean of 5). |

rRNA is present in *E. coli* in approximately 6 fold greater quantities than tRNA. Therefore, allowing for this, an average composition of *Streptomyces* RNA is:

| A(%) | G(%) | C(%) | U(%) |
|---|---|---|---|
| 21.8 | 32.9 | 26.3 | 19.2 |

with limits of 58% to 63% GC mol %.

# Appendix C

**Fatty acid composition of *S. lividans* for use in precursor tables of *S. coelicolor*.**

The lipid composition of *E. coli* (Neidhardt, 1987) was calculated on the basis of an average $C_{16}$ fatty acid. This calculation was also used to determine the fatty acid compostion of *S. coelicolor*.

According to U. Gräfe, G. Reinhardt and D. Noak (submitted to Journal of Actinomycetes), *S. lividans* biomass (as dry weight) is composed of 5.5% lipid. *S. lividans* is very closely related to *S. coelicolor* (Kieser *et al.*, 1992). Therefore, for the purpose of flux determinations, the lipid composition of *S. coelicolor* was assumed to be equivalent to that of *S. lividans*. The following calculations are based on the data from Gräfe *et al.* (personal communication).

Lipid composition of 1g dry weight *S. lividans* biomass (55mg total lipid):

| | | lipid | | fatty acid | |
|---|---|---|---|---|---|
| | % | mg | µmole[a] | chains | mmole |
| neutral lipid | 60.4 | 33.22 | 38.9 | 3 | 0.117 |
| glycolipid | 12.1 | 6.66 | 8.5 | 2 | 0.017 |
| phospholipid | 20.9 | 11.50 | 16.3 | 2 | 0.033 |
| glycerol | | | 63.7 | | |
| total $C_{16}$ fatty acid | | | | | 0.167 |

a, assuming an average fatty acid chain of $C_{16}$ has a molecular weight of 255.

Composition of lipid species:

| | branched chain fatty acids | | amino acid precursors(mmol) | |
|---|---|---|---|---|
| | iso[b](%) | anteiso[c](%) | leucine | isoleucine |
| neutral lipid | 86.2 | 9.7 | 0.1 | 0.011 |
| glycolipid | 83.3 | 11.2 | 0.014 | 0.002 |
| phospholipid | 82.9 | 13.1 | 0.027 | 0.004 |
| | | | 0.141 | 0.017 |

b, iso- branched chain fatty acids have leucine as a pecursor; c, anteiso-branched chain fatty acids have isoleucine as a precursor. Gräfe *et al.* measured an average of 95.4% branched fatty acids. This is higher than that cited for *S. coelicolor* (84%; Kaneda, 1991).

# Appendix D

## Determination of amount of $CO_2$ produced in effluent gas.

The following equations were taken, with kind permission, from Hamilton (1972).

Gaseous $CO_2$

$CO_2$ produced in effluent gas was measured as a percentage of the gas flowing through the $CO_2$ analyzer. Assuming a respiratory quotient of approximately 1.0 and that the flow of air in is equivalent to the flow of air out,

$$\text{volume } CO_2 \ (l.h^{-1}) = \frac{CO_2\%}{100} \times \text{flow rate}$$

1mole $CO_2$ is equivalent to 22.4litres at standard temperature and pressure. Therefore, assuming P=1.0atm.,

$$\text{Gaseous } CO_2 \ (mol.h^{-1}) = \frac{CO_2\% \times \text{flow rate}}{100} \times \frac{273}{T^oA \times 22.4}$$

To calculate the rate of production of $CO_2$, the culture volume is taken into consideration:

$$\text{Gaseous } CO_2 \text{ production } (mol.l^{-1}.h^{-1}) = \frac{CO_2\% \times \text{flow rate} \times 273}{100 \times T^oA \times 22.4 \times \text{culture vol.}}$$

$CO_2$ in solution

At 30°C (standard T and P),
volume of $CO_2$ absorbed by 1volume of $H_2O$ = $0.665ml.ml^{-1}$.
(Handbook of Chemistry and Physics, 41st edition, 1959)

a)

$$\frac{0.665ml.ml^{-1}}{22.4} \ (l.l^{-1}) = \frac{0.0297mol.l^{-1} \times 1000}{100}$$

$$= 0.297mM \ CO_2 \text{ at } 1\% \ CO_2 \text{ in gas.}$$

b) 1litre $H_2O$ contains 1.257g = 28.56mM $CO_2$ at 100%

$$= 0.286mM \ CO_2 \text{ at } 1\% \ CO_2 \text{ in gas.}$$

Mean of a) and b) is 0.292mM $CO_2$ in solution at 1% $CO_2$ in gas.
Therefore,

$$[CO_2] = CO_2\ \%\ x\ 0.292mM.$$

In solution, however,

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

which is pH dependent according to the Henderson Hasselbach equation:

$$pH = pK_a + \log \frac{[HCO_3^-]}{[CO_2]}$$

therefore,

$$\log \frac{[HCO_3^-]}{[CO_2]} = pH - pK_a$$

According to "Data for Biochemical Research," the dissociation constant ($k_a$) for carbonic acid at 30°C is $4.71 \times 10^{-7}$.
Therefore,

$$\log K_a = -6.327$$
$$-\log K_a = 6.327 = pK_a$$

At pH7.0,

$$\log \frac{[HCO_3^-]}{[CO_2]} = 7.00 - 6.327$$
$$= 0.673$$

Therefore,

$$\frac{[HCO_3^-]}{[CO_2]} = 4.710, \text{ at } 1\%\ CO_2 \text{ in air}$$

and,

$$\frac{[HCO_3^-] + [CO_2]}{[CO_2]} = 5.710$$

Total dissolved $CO_2$, at 30°C and 1atm. P when partial pressure in the gas phase is 1%:

$$[HCO_3^-] + [CO_2] = 5.71 \times [CO_2]$$
$$= 5.71 \times 0.292mM$$
$$= 1.667mM.$$

A table was constructed to calculate the concentration of total dissolved $CO_2$ at 30°C at different pH measurements (table D1). The range of pH measurements included those observed in the *S. coelicolor* fermentation.

**Table D1. Effect of pH on $[HCO_3^-]$ and $[CO_2]$ at 30°C**

| pH | $\log \frac{[HCO_3^-]}{[CO_2]}$ | $\frac{[HCO_3^-]}{[CO_2]}$ | $\frac{[HCO_3^-] + [CO_2]}{[CO_2]}$ | $[HCO_3^-] + [CO_2]$ |
|---|---|---|---|---|
| 6.8 | 0.473 | 2.972 | 3.972 | 1.16 |
| 6.9 | 0.573 | 3.741 | 4.741 | 1.384 |
| 6.95 | 0.623 | 4.197 | 5.197 | 1.518 |
| 7.0 | 0.673 | 4.710 | 5.710 | 1.667 |
| 7.1 | 0.773 | 5.929 | 6.929 | 2.023 |
| 7.2 | 0.873 | 7.464 | 8.464 | 2.472 |
| 7.25 | 0.923 | 8.375 | 9.375 | 2.738 |
| 7.3 | 0.973 | 9.397 | 10.397 | 3.036 |
| 7.4 | 1.073 | 11.830 | 12.830 | 3.746 |
| 7.5 | 1.173 | 14.894 | 15.894 | 4.641 |
| 7.6 | 1.273 | 18.750 | 19.750 | 5.767 |
| 7.65 | 1.323 | 21.038 | 22.038 | 6.435 |
| 7.7 | 1.373 | 23.605 | 24.605 | 7.184 |
| 7.8 | 1.473 | 29.717 | 30.717 | 8.969 |

The values in the columns were used to calculate the total concentration of $CO_2$ in solution at the different pH values in the fermenter and at the different percentage $CO_2$ contents of air.

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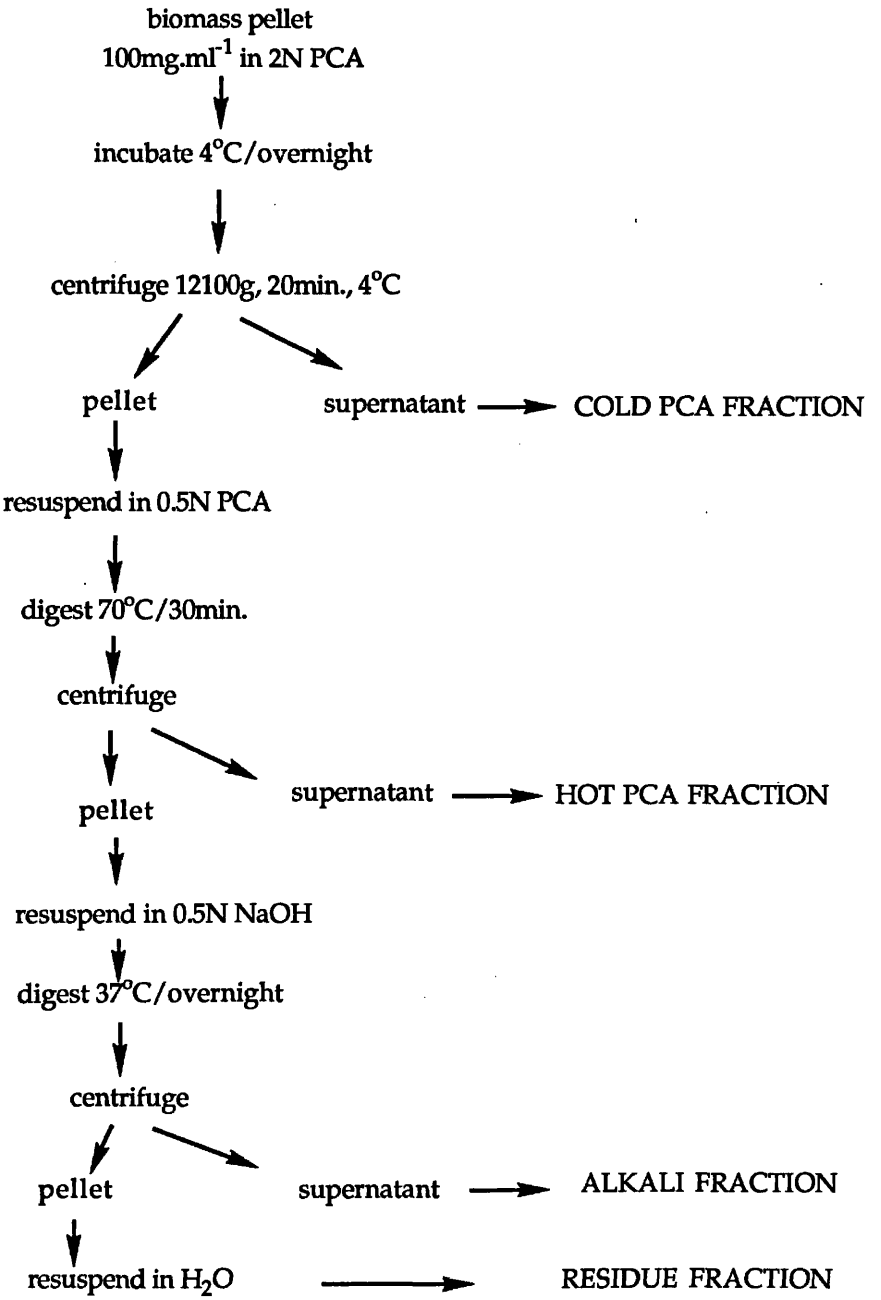
## Addendum

## Flow charts of fractionation methods 1 and 2

a. Method 1



**b.** Method 2.