THE EXAMINATION OF ORGANIC ACID PRODUCTION
DURING THE GROWTH OF *STREPTOMYCES LIVIDANS* TK24

A thesis submitted for the degree of Doctor of Philosophy
to the University of London
by

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ABSTRACT

Organic acid excretion by *Streptomyces lividans* TK24 lowers the culture medium pH and may interfere with growth and recombinant product integrity. The acids excreted were identified by HPLC and enzymic assays as pyruvate and α-ketoglutarate.

Acid excretion occurred during growth on glucose in defined medium, but was dependent on the nitrogen source employed. With nitrate as the sole nitrogen source, high levels of pyruvate and traces of α-ketoglutarate were detected. Carbon from D-[U-\(^{14}\)C]glucose was converted to both acids. With a selected amino acid as the sole nitrogen source, less pyruvate and more α-ketoglutarate was excreted. The sum of maximum acid levels was greater than in glucose-nitrate media. Carbon from both labelled glucose and amino acids was converted to the acids. With ammonium as the sole nitrogen source, no acids were produced. Cultures supplied with mixed organic and inorganic nitrogen sources exhibited a reversion of the acid production pattern as if the organic source were absent.

Experiments with alternative carbon sources showed that starch, maltose and glycerol supported acid production. Levels of excreted acids were higher when the carbon sources were combined with an organic nitrogen source compared to nitrate. Fructose, sucrose and dextrin did not support high growth or any detectable acid overproduction.

Acid accumulation occurred in complex MEP medium (1% malt extract broth, 1% peptone, 2% glycerol), and in media containing the constituent carbon sources, malt extract and malt extract broth, with defined nitrogen sources. The acid production patterns were generally equivalent to the most similar defined combinations. It was concluded that acid production was supported by the principal carbon sources and influenced by the amino acids in the peptones. An ammonium supplement reduced acid production by 85%.

In most media, acids were reassimilated towards the end of rapid growth and during the stationary phase. Conversion of consumed carbon to undesirable acid by-products was greater in defined media (around 11%) than complex media (around 6%).
ACKNOWLEDGEMENT

I would like to thank my supervisors, Dr. Andrew Ison and Dr. John Ward for their help and guidance throughout this project.

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Finally, very many thanks to my parents for their unending support, to David who was always patient, and to Michael for encouraging me towards the end.
For my parents and David;
and for my Grandfather who did not live to see this completed.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>g, mg, µg, ng</td>
<td>grams, milligrams, micrograms, nanograms</td>
</tr>
<tr>
<td>L, mL, µL</td>
<td>litres, millilitres, microlitres</td>
</tr>
<tr>
<td>M, mM</td>
<td>molar, millimolar</td>
</tr>
<tr>
<td>mm, nm</td>
<td>millimetres, nanometres</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>RI</td>
<td>refractive index</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>DOT</td>
<td>dissolved oxygen tension</td>
</tr>
<tr>
<td>OUR</td>
<td>oxygen uptake rate</td>
</tr>
<tr>
<td>CER</td>
<td>carbon dioxide evolution rate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>H⁺</td>
<td>hydrogen ions</td>
</tr>
<tr>
<td>Ci</td>
<td>curies (1 µCi = 37 kBq)</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

The aim of this project was the characterisation of organic acid production by Streptomyces lividans when grown in media containing a range of defined and complex carbon and nitrogen sources. The plasmid-free strain *S. lividans* TK24 is a popular host for the cloning of autologous and heterologous genes, and expression and secretion of the corresponding products (see later sections for examples). Growth and production by this organism may be affected by the growth conditions (Lee & Lee, 1994), especially pH. The medium pH will fluctuate throughout a fermentation with organic acid excretion and reuse.

Previous studies have shown that *S. lividans* TK24 produces organic acids during rapid growth in a complex medium containing malt extract, peptone and glycerol (Wrigley-Jones, 1991). Acidification reduced the medium pH as low as 2.0-3.0 and interfered with growth. The acids were not identified during this preliminary study.

Other studies using *S. lividans* TK24 have shown that pH fluctuations reduce the yield and activity of recombinant products (Erpicum *et al.*, 1990). Reducing acid formation by the parent strain *S. lividans* 66 improved the efficiency of conversion of precursors to desired products (Payne *et al.*, 1990).

It is of interest to know the identity of the organic acids produced by *S. lividans* TK24, the conditions resulting in their production and their sources within the central metabolic pathways. The information gained from this project will potentially be of future use in improving the yield of recombinant products from this organism.
1.1 The Importance of *Streptomyces* and *Streptomyces lividans*

1.1.1 *Streptomyces* Development and Industrial Importance

The aim of this section is to provide an introduction to the bacterial genus *Streptomyces* and the natural products, typically antibiotics, which make these organisms important in industry. In addition, certain streptomycete strains such as *Streptomyces lividans* have been found useful as hosts for the cloning and expression of autologous and heterologous products, thus increasing their industrial potential.

1.1.1.1 Streptomycete Products

*Streptomyces* are Gram-positive aerobic soil bacteria. They are multicellular and have an unusual filamentous structure. Colonies develop from spores as a mass of mycelia called the substrate or vegetative mycelium. The mycelium is composed of branched hyphae which obtain nutrients from the organic matter in soil via the action of extracellular enzymes. After a period of vegetative growth, the colony undergoes morphological differentiation to form a spore-bearing aerial mycelium. This structural differentiation is found to be accompanied by physiological changes. The metabolism of the colony switches from a primary phase to meet the requirements of vegetative growth, to a secondary stage during which secondary metabolites including antibiotics are produced (Hopwood, 1988; Chater, 1989; Hopwood *et al.*, 1992).

Morphological and physiological changes are temporally associated and seem to be regulated by complex, inter-connected genetic mechanisms (Hopwood, 1988; Chater, 1989). Regulatory mechanisms are activated in response to signals which may include nutritional depletion. This will be considered in more detail in Section 1.3.

Secondary metabolites are produced during the 'transition phase' from the production of vegetative to aerial mycelia, and it is possible that they are involved in morphological differentiation itself. It has also been suggested that some may act to defend the vulnerable colony from attack by motile micro-organisms as parts of it lyse to provide nutrients for aerial mycelium formation (Hopwood, 1988; Chater, 1989).

It has recently been said that 'Streptomyces qualify as the most important group of industrial micro-organisms' due to the diverse range of secondary metabolites produced by this genus (C.W. Chen (1995) *Trends In Biotechnology* 13 157-160). Several authors have noted that over 60% of antibiotics of microbial origin are obtained
from *Streptomyces* (Piret & Demain, 1988; Wallace *et al.*, 1992; Anne & Van Mellaert, 1993), including β-lactams, polyketides, aminoglycosides and peptide antibiotics. A large number of these are commercially important and are used in human and veterinary medicine. It is often found that one strain will produce a number of antibiotics (Piret & Demain, 1988). For example, *Streptomyces coelicolor* produces four antibiotics (the red-blue pigment actinorhodin, the red pigment undecylprodigiosin, unpigmented methylenomycin and calcium-dependent antibiotic) and one secondary metabolite, a γ-butyrolactone of unclear function but capable of influencing antibiotic production (Hopwood, 1988).

Some secondary metabolites have other biologically active properties (*e.g.* anti-tumour drugs for cancer treatment and immunosuppressants used after organ transplants). Many are used in agriculture as herbicides, antihelmintics, fungicides and insecticides (Piret & Demain, 1988; Hopwood *et al.*, 1992; Gusek & Kinsella, 1992). Certain naturally produced extracellular enzymes are also of interest for use in biotechnology processes, medical applications and for recycling waste materials, including α-amylases, ligninases, xylanases, cellulases, chitinases, proteases and lipases. The ability of streptomycetes to secrete these products is at the heart of their importance (Hutchinson, 1987; Piret & Demain, 1988; Gusek & Kinsella, 1992).

### 1.1.1.2 Genetic Manipulation

In recent years it has become clear that it is possible to manipulate *Streptomyces* strains in order to improve the yield of existing products or to produce novel secondary metabolites. Certain strains such as *Streptomyces lividans* (used throughout this project) are proving useful for the cloning and expression of heterologous proteins from diverse prokaryotic and eukaryotic sources (Wallace *et al.*, 1992). Many of the reported examples have been embraced by the emerging science of metabolic engineering. This has been defined by Bailey (1991) as 'the improvement of cellular activities by the manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology'. Bailey (1991) notes that existing metabolic pathways are not optimised in the ways required by industry. Metabolic restructuring by genetic manipulation enables the improvement in yield of secondary metabolites and proteins and the ability to obtain new products.
A selection of foreign (heterologous) antibiotics and proteins cloned into *S. lividans* are shown in Tables 1.1 and 1.2.

<table>
<thead>
<tr>
<th>ANTIBIOTIC</th>
<th>SOURCE</th>
<th>REFERENCE</th>
</tr>
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<tbody>
<tr>
<td>actinorhodin</td>
<td><em>Streptomyces coelicolor</em></td>
<td>Malpartida &amp; Hopwood, 1984</td>
</tr>
<tr>
<td>undecylprodigiosin</td>
<td><em>Streptomyces coelicolor</em></td>
<td>Malpartida et al., 1990</td>
</tr>
<tr>
<td>erythromycin</td>
<td><em>Streptomyces erythreus</em></td>
<td>Stanzak et al., 1986</td>
</tr>
<tr>
<td></td>
<td><em>(Saccharopolyspora erythrea)</em></td>
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**Table 1.1:** Selected examples of whole or part antibiotic biosynthetic pathways cloned into *S. lividans*.

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>SOURCE</th>
<th>REFERENCE</th>
</tr>
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<tbody>
<tr>
<td>agarase</td>
<td><em>Streptomyces coelicolor</em></td>
<td>Kendall &amp; Cullum, 1984</td>
</tr>
<tr>
<td>α-amylase</td>
<td><em>Streptomyces hygroscopicus</em></td>
<td>McKillop et al., 1986</td>
</tr>
<tr>
<td>α-amylase</td>
<td><em>Streptomyces limosus</em></td>
<td>Long et al., 1987</td>
</tr>
<tr>
<td>α-amylase</td>
<td><em>Streptomyces venezuelae</em></td>
<td>Virolle et al., 1988</td>
</tr>
<tr>
<td>α-amylase</td>
<td><em>Streptomyces thermoviolaceus</em></td>
<td>Bahri &amp; Ward, 1990</td>
</tr>
<tr>
<td>α-amylase</td>
<td><em>Thermomonospora curvata</em></td>
<td>Petricek et al., 1989</td>
</tr>
<tr>
<td>tendamistat</td>
<td><em>Streptomyces tendae</em></td>
<td>Koller &amp; Riess, 1989</td>
</tr>
<tr>
<td>proinsulin</td>
<td><em>Macaca fascicularis</em></td>
<td>Koller et al., 1989</td>
</tr>
<tr>
<td>interleukin-2</td>
<td><em>Homo sapiens</em></td>
<td>Bender et al., 1990a</td>
</tr>
<tr>
<td>hirudin</td>
<td><em>Hirudo medicinalis</em></td>
<td>Bender et al., 1990b</td>
</tr>
<tr>
<td>VHB</td>
<td><em>Vitreoscilla</em></td>
<td>Magnolo et al., 1991</td>
</tr>
<tr>
<td>CD4</td>
<td><em>Homo sapiens</em></td>
<td>Fornwald et al., 1993</td>
</tr>
<tr>
<td>tyrosinase</td>
<td><em>Streptomyces antibioticus</em></td>
<td>Katz et al., 1983</td>
</tr>
<tr>
<td>parathion hydrolase</td>
<td><em>Flavobacterium</em></td>
<td>Steiert et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Payne et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DelaCruz et al., 1992</td>
</tr>
<tr>
<td>β-lactamase</td>
<td><em>Streptomyces sp.</em></td>
<td>Erpicum et al., 1990</td>
</tr>
<tr>
<td>carboxypeptidase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.2:** Selected examples of heterologous proteins cloned into *S. lividans*.

The cloning of antibiotics is facilitated by the fact that the genes for entire pathways tend to be clustered together, for example the actinorhodin (act) and undecylprodigiosin (red) clusters in *S. coelicolor* (Bibb, 1996). Clusters contain biosynthetic, regulatory and product resistance genes (Hopwood, 1988; Chater, 1990; Hopwood *et al*., 1992; Wallace *et al*., 1992). The examples in Table 1.1 include whole and part pathways. Cloning has recently allowed the generation of novel products, as predicted by Hopwood *et al.* (1983a). The same group (Hopwood *et al*., 1985b)
demonstrated that the transfer of act genes from S. coelicolor into strains producing similar antibiotics yielded the novel hybrid products mederrhodin and dihydrogranatirhodin.

From Table 1.2, it can be seen that a very diverse range of proteins have been successfully cloned into and expressed by S. lividans, demonstrating the importance and versatility of this strain. The strain used for the majority of these examples was TK24, which was used for this project. The advantages of expression in S. lividans include elevated levels of production [e.g. Kendall & Cullum (1984) reported that recombinant S. lividans exhibited 6 to 500 times more agarase activity than the S. coelicolor parent]; expression of industrially-important materials such as α-amylase; expression of proteins from both unrelated prokaryotes (e.g. Payne et al., 1990) and even eukaryotes (e.g. Koller et al., 1989); and improvement of growth characteristics [Magnolo et al. (1991) found that expression of Vitreoscilla haemoglobin by S. lividans improved biomass yield].

The major advantage of a strain such as S. lividans over a more traditionally used bacterium such as Escherichia coli is the ability of the former to secrete proteins into the extracellular medium (Chater & Hopwood, 1982; Wallace et al., 1992). Further advantages specific to S. lividans will be considered in Section 1.1.2.

The expression of recombinant products by streptomycetes may be affected by the growth conditions. The active production of organic acids reduces the pH of the extracellular medium. This can affect the growth of the organism and product yield and activity (Doull & Vining, 1990a; Payne et al., 1990; Wrigley-Jones et al., 1993). A metabolic imbalance through the glycolytic and tricarboxylic acid cycle pathways may lead to the build-up of toxic by-products as well as the conversion of substrates/precursors to unwanted by-products (DelaCruz et al., 1992). Acidification and pH effects are discussed in Sections 1.2 and 1.4.

The extracellular pH, combined with other parameters such as medium type and culture conditions, may affect the hyphal morphology (Prosser & Tough, 1991). S. lividans is prone to forming pellets in liquid culture (Whitaker, 1992). The interior biomass of pellets may become deprived of nutrients and oxygen, which can affect product synthesis (Bushell, 1988). It is possible that these oxygen-deprived cells also secrete TCA cycle-associated organic acids. These factors are discussed in Section 1.4.
Certain nutrients are thought to interfere with other metabolic pathways, notably glucose, ammonium ions and phosphate (Demain, 1992). Suppressed pathways are active only after depletion of the interfering carbon, nitrogen or phosphate source. Carbon and nitrogen sources may be involved in acid release (Ahmed et al., 1984). Also, certain medium combinations designed to prevent pH fluctuations (Bader, 1986) may affect catabolite control, for example phosphate buffers (Aharonwitz & Demain, 1977). The phenomenon of catabolite regulation is considered in Section 1.3.

In conclusion, *Streptomyces* have great commercial importance as the producers of a wide range of industrially, medicinally and agriculturally useful secondary metabolites. In addition, genetic manipulation techniques have been developed allowing the rational improvement of product yields and the generation of novel products. Novel antibiotics may be more potent, have a wider spectrum of activity, and can be used to bypass resistance developed by pathogens to older types (Hopwood et al., 1983a). Increased titres of antibiotics and enzymes will improve the costs of industrial processes (Hutchinson, 1987; Gusek & Kinsella, 1992). It has also been noted that the use of an alternative host may mean that the new host is easier to handle, more robust and able to use cheaper and/or a more extensive range of substrates (Bailey, 1991).

However, recombinant products may be affected by a number of interacting internal and external factors, including acid production, carbon, nitrogen and phosphate source effects and other parameters such as pH and morphology. These effects should be taken into account when manipulating *Streptomyces* strains.
1.1.2 Streptomyces lividans

The theme of this project is the characterisation of acid production by *Streptomyces lividans*. This section has two parts: (1) a review of the advantages and popularity of *S. lividans* as a host for genetic manipulation; and (2) a discussion of the antibiotic pigment-producing capability of this strain. Examples of recombinant products were given in Section 1.1.1.

1.1.2.1 Advantages of *Streptomyces lividans* as a Recombinant Host

*S. lividans* has been described as the ‘workhorse’ of streptomycete recombinant DNA technology (Hutchinson, 1987). As a Gram-positive bacterium, it shares with other streptomycetes several advantages over the Gram-negative bacterium *E. coli*. Several additional characteristics make this strain more suitable for cloning than other *Streptomyces* species (reviewed recently by Gusek & Kinsella, 1992).

*Streptomyces* are able to efficiently secrete a wide range of naturally-occurring products. The secretion of extracellular enzymes is important during the life cycle for the uptake of nutrients from the soil growth medium. In industry, the ability to secrete compounds simplifies downstream processing for the recovery of pure, active recombinant products (Gusek & Kinsella, 1992; Anne & Van Mellaert, 1993; Kleman & Strohl, 1994). In comparison, *E. coli* cannot export proteins as easily due to the presence of an outer cell membrane and proteins are often retained in the cell in inactive forms. This can complicate downstream processing and makes extraction and activation of the product impractical at larger scales (Chater & Hopwood, 1982; Anne & Van Mellaert, 1993). However, for *Streptomyces* to succeed as viable alternatives to *E. coli*, similar or higher yields of recombinant proteins as this organism must be achieved (Anne & Van Mellaert, 1993). In 1992, Wallace *et al.* noted that few groups using *S. lividans* had been able to achieve protein titres over 100mg·L\(^{-1}\), but that methods were being developed to increase yields [for example, the use of promoter sequences (Koller *et al.*, 1989) and feeding of substrates and precursors (Payne *et al.*, 1990)]. By creating a fusion gene of the human T-cell receptor CD4 and the promoter and signal sequences from a *S. longisporus* serine protease inhibitor, Fornwald *et al.* (1993) obtained yields of up to 300mg·L\(^{-1}\) of active CD4 derivatives.

The majority of *Streptomyces* species are non-pathogenic and very few are suspected or proved to cause disease in humans, animals or plants (Gusek & Kinsella, 1992). *E. coli* is a human commensal and has a number of close relatives which are
pathogenic. Also, the outer membrane of \textit{E. coli} contains toxins which must be removed from pharmaceutical products, again complicating downstream processing (Chater \& Hopwood, 1982; Kleman \& Strohl, 1994).

\textit{S. lividans} lacks an endonuclease restriction barrier against unmodified foreign DNA (Chater \& Hopwood, 1982; Hutchinson, 1987). Most other streptomycetes, including the genetically well-characterised \textit{S. coelicolor}, show strong restriction against foreign DNA. \textit{S. lividans} also does not restrict DNA from other streptomycetes, while \textit{S. coelicolor} will restrict DNA even from its close relative \textit{S. lividans} (Hopwood \textit{et al.}, 1983b; Anne \& Van Mellaert, 1993). This feature makes \textit{S. lividans} a good host for the intergeneric transfer of DNA (Gusek \& Kinsella, 1992; Anne \& Van Mellaert, 1993). In addition, \textit{S. lividans} is easy to grow, grows and sporulates quickly, is transformable with exogenous DNA and is generally more convenient to use than \textit{S. coelicolor} (Hopwood \& Chater, 1982; Hopwood \textit{et al.}, 1983b; Hutchinson, 1987).

\textit{S. lividans} is able to use a wide range of heterologous promoters for protein synthesis (Gusek \& Kinsella, 1992). \textit{Streptomyces} DNA has a high bias of guanosine and cytosine residues (mean 74\%\; ; Bibb, 1996) which may explain why few \textit{Streptomyces} genes can be expressed by \textit{E. coli}. This bias does not prevent \textit{S. lividans} from expressing genes from other prokaryotes including \textit{E. coli}, \textit{Serratia marcescens} and \textit{Bacillus} spp. (Hutchinson, 1987; Piret \& Demain, 1988). This was demonstrated by Stanzak \textit{et al.} (1986) who found that genes from \textit{S. erythrea} were expressed by \textit{S. lividans} TK23 but not \textit{E. coli}.

Wallace \textit{et al.} (1992) noted that \textit{Streptomyces} are known to glycosylate antibiotics. Anne \& Van Mellaert (1993) subsequently noted that \textit{S. lividans} may be able to glycosylate proteins, which is important for the correct processing of eukaryotic products. \textit{S. lividans} is the host for a range of cloning vectors (Chater \& Hopwood, 1982; Hopwood \& Chater, 1982). These vary in features such as copy number, insertion sites and colour markers, enhancing the number of cloning tools available. The most commonly used is the multicopy vector pIJ702, which contains thiostrepton resistance (\textit{tsr}) and the tyrosinase gene (\textit{mel}) for selection of transformants (Katz \textit{et al.}, 1983). The \textit{S. lividans}/pIJ702 system is highly stable, even in the absence of thiostrepton selection or the presence of the mutagen ethidium bromide (Wallace \textit{et al.}, 1992; Gusek \& Kinsella, 1992). However, pIJ702 is unsuitable for large DNA inserts \textit{e.g.} Malpartida \& Hopwood (1984) were unable to use pIJ702 to clone the \textit{act} cluster from \textit{S. coelicolor} into \textit{S. parvulus}. 

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Hopwood et al. (1983b; also Kieser et al., 1982) reported the development of a series of plasmid-free derivatives from the parent strain *S. lividans* 66 (John Innes stock number 1326) which carries plasmids SLP2 and SLP3. These include the strain TK24 which was used for this project. Plasmid-free strains are useful for cloning experiments since the interference of host plasmids with introduced vector-determined phenotypes is minimised.

It has been reported that in contrast to other streptomycetes, *S. lividans* secretes insignificant amounts of extracellular proteases. These are undesirable as they can degrade secreted recombinant products (Gusek & Kinsella, 1992; Kleman & Strohl, 1994). Several authors report having no problems with proteases (e.g. Erpicum et al., 1990; Payne et al., 1990). When proteases are present it is possible to prevent adverse effects. Aretz et al. (1989) describe the use of certain metal ions (nickel and zinc at 1mM concentrations) to effectively inhibit the action of a protease which specifically degraded a secreted recombinant fusion protein (Koller et al., 1989), although other metal ions such as calcium and magnesium stimulated protease activity. Fornwald et al. (1993) achieved high-level expression of CD4 derivatives after controlling protease action or formation by the addition of casamino acids to the medium. Both solutions allow a simple alternative to the creation of mutant, protease-deficient hosts.

*S. lividans* has been reported to produce the antibiotic pigments actinorhodin and undecylprodigiosin. These may interfere with the analysis of foreign gene expression (Hutchinson, 1987), as demonstrated by Malpartida et al. (1990). However, pigment products may prove useful for the study of genetic control and the effect of growth conditions in this strain. Also, it has been reported that expression of the pigments is low or absent in the growth conditions commonly used (Horinouchi & Beppu, 1984). Pigment production by *S. lividans* is discussed in more detail in the following section.

### 1.1.2.2 Antibiotic Products

*S. lividans* and its close relative *S. coelicolor* are regarded as sub-strains of *S. violaceoruber* (Gusek & Kinsella, 1992). Members of this group typically produce pH-sensitive pigments (which are not melanoid pigments) which cause the underneath of agar-grown colonies to appear blue or violet. Both strains produce actinorhodin (blue or red with alkaline or acidic conditions respectively) and undecylprodigiosin (a red cell-wall associated pigment) (Horinouchi et al., 1983; Hobbs et al., 1990). Neither are of commercial importance but both are of scientific importance as colour indicators for
genetic analysis (Hopwood et al., 1992). Natural pigment products of S. lividans TK24 could potentially be used to study the effects of organic acid excretion. This sub-section reviews what is known of pigment production and other factors controlling it.

*S. coelicolor* produces large amounts of both pigments, but under commonly-used culture conditions pigment expression by *S. lividans* occurs at low levels (Horinouchi et al., 1986, 1989). For example, Horinouchi et al. (1983) and Horinouchi & Beppu (1984) observed that agar colonies of *S. lividans* TK21 were only slightly coloured after 14 days growth (d).

It has been reported that the cloning of pathway-specific positive regulatory genes from the *act* and *red* clusters of *S. coelicolor* results in overproduction of actinorhodin and undecylprodigiosin by *S. lividans* (Malpartida et al., 1990). In addition, a gene from *S. coelicolor* which pleiotropically regulates secondary metabolite biosynthesis in both strains has been cloned. This gene was originally thought to be a locus called *afsB*, but has now been identified as *afsR* (Stein & Cohen, 1989; Bibb, 1996).

Horinouchi et al (1983) and Horinouchi & Beppu (1984) found that the presence of this cloned fragment from *S. coelicolor* caused high levels of pigment expression by *S. lividans* TK21. The pigments were confirmed to be actinorhodin and undecylprodigiosin and were produced at such high levels that agar cultures turned deep red within 3d and dark blue after prolonged incubation. Liquid cultures became blue after 3 to 4 days. The authors concluded that the *afsB* (*afsR*) fragment positively controlled expression of biosynthetic genes in *S. coelicolor* and was able to enhance expression of dormant or 'silent' genes in *S. lividans*.

Subsequently, Horinouchi et al. (1986) revealed that the amino acid sequence estimated from the nucleotide sequence of the *afsB(R)* gene contained regions resembling other known DNA-binding proteins, suggesting that the *afsB(R)* product acted as a transcriptional activator. This was supported by the observation that *S. lividans* TK21 lacking the cloning vector, or containing vector without the *afsB(R)* insert contained low levels of *act* transcripts. Strain TK21 cloned with *afsB(R)* DNA contained an elevated level of *act* transcripts and had correspondingly increased expression of actinorhodin.

Sequences homologous to *afsB(R)* do occur in *S. lividans* (Horinouchi et al., 1986). It is unclear why *afsR* present on a vector can enhance pigment production in low level expressing strains of *S. lividans* when the chromosomal *afsR* cannot. A likely
reason would be a mutation to the *afsR* region in *S. lividans* (Stein & Cohen, 1989). Horinouchi *et al.* (1986) noted that *S. lividans* TK21 and mutant derivatives are able to spontaneously produce high levels of pigment under certain growth conditions, for example when grown on excess sucrose. They suggested that pigment production by *S. lividans* may or may not be regulated by chromosomal *afsR* but may be totally dependent on physiological conditions. It has been noted elsewhere than antibiotic production by streptomycetes may be a response to physiological stress. Hobbs *et al.* (1992) suggested that methylenomycin production by *S. coelicolor* may be due to pH stress; conversely Horinouchi *et al.* (1989) noted that *S. coelicolor* *afsB'* mutants synthesised reduced levels of methylenomycin, indicating control of the synthesis of this product by *afsB(R)*.

It has recently been noted that *afsR* may be part of a two-component regulatory system with a gene called *afsK* (Virolle & Gagnat, 1994). Another possible two-component regulatory system in *S. coelicolor* comprising genes *afsQ1* and *afsQ2* has been identified by Ishizuka *et al.* (1992). Cloned *afsQ1* conferred similar effects as cloned *afsR* when introduced into *S. lividans*, and sequences homologous to *afsQ1/afsQ2* were identified in *S. lividans*. These regulatory systems are found to be similar to known two-component systems and signal transduction pathways in other bacteria (Ishizuka *et al.*, 1992; Virolle & Gagnat, 1994).

Bibb (1996) noted that the product of *afsR* (AfsR) was found to be a homologue of the pathway-specific regulatory proteins for the *act* (ActII-ORF4) and *red* (RedD) clusters. Analysis showed *afsR* to be a true pleiotropic regulatory gene, although its mode of action remains unclear since mutations in *afsR* and the pathway-specific regulatory genes did not affect each other. It is suspected that AfsR might interact with pathway-specific regulatory proteins after transcription.

Recently, Carbo *et al.* (1995) reported that a DNA fragment from the unrelated prokaryote *Mycobacterium bovis* is capable of inducing actinorhodin synthesis in both *S. lividans* and *S. coelicolor*. Actinorhodin levels from *S. lividans* were increased fivefold by 6 days of growth, and 28-fold from *S. coelicolor* by 8 days. These results demonstrate both that the DNA region cloned from *M. bovis* had similar activity to *afsR* and that *S. lividans* is capable of expressing heterologous DNA sequences. Further examples of heterologous gene expression were given in Section 1.1.1.
1.2 Acid Production During Bacterial Growth

1.2.1 Organic Acid Production by Streptomyces

Since the aim of this project is the characterisation of organic acid production by *Streptomyces lividans* TK24, this section contains a review of the current known examples of acid production by *Streptomyces* species.

The excretion of large amounts of acidic metabolites into the extracellular medium has been observed in cultures of a variety of *Streptomyces* species grown in defined or complex media containing glucose or other rapidly-used carbon sources. These include *Streptomyces griseus* (Hockenhull *et al.*, 1954), *Streptomyces aureofaciens* (Doskocil *et al.*, 1959), *Streptomyces venezuelae* (Ahmed *et al.*, 1984), *Streptomyces alboniger* (Surowitz & Pfister, 1985), *Streptomyces peucetius* (Dekleva & Strohl, 1987), *Streptomyces coelicolor* (Hobbs *et al.*, 1992) and *Streptomyces lividans* 66 (Payne *et al.*, 1990; DelaCruz *et al.*, 1992). In the majority of these examples, the principal organic acids produced were α-keto acids associated with the glycolytic and tricarboxylic acid (TCA) cycle pathways (*i.e.* organic acids containing a ketone group (-C=O) adjacent to the functional carboxyl group (-COOH) in each molecule). Figure 1.1 and Figure 1.2 show schematic diagrams of the reactions involved in glycolysis and the TCA cycle, including the positions of carboxylic acid intermediates.

One of the earliest reports of acid excretion by a streptomycete is given by Hockenhull *et al.* (1954). During studies on glucose utilisation, the authors noted that when *S. griseus* was grown in a defined medium under restricted aeration, the organism produced a large amount of lactic acid by around 72 hours of growth (h). This would be expected under anaerobiosis, following the conversion of pyruvate to lactate by lactate dehydrogenase which is a common pathway in micro-organisms.

However, it was also observed that during early rapid growth (20-30h) on glucose in either defined or complex media and in aerobic conditions, the organism excreted significant quantities of α-keto acids. These ranged from 0.38mg-mL\(^{-1}\) keto acid in complex meat extract medium to 0.198mg-mL\(^{-1}\) keto acid in defined medium. In each case pyruvate was found to be the major acid product (up to 90%) with the remainder made up of traces of other substances including α-ketoglutarate and oxaloacetate.
Figure 1.1: Diagram showing the major reactions of the glycolytic pathway and points of entry of a number of carbohydrates (after Stryer, 1995).
pyruvate + CoA + NAD\(^+\) \[\rightarrow\] acetyl CoA + CO\(_2\) + NADH

Figure 1.2: Schematic representation of the conversion of pyruvate to acetyl CoA and the major reactions of the tricarboxylic acid cycle (after Stryer, 1995).
Doskocil et al. (1959) found that \textit{S. aureofaciens} also produced pyruvate during early rapid growth in a complex medium containing sucrose as the primary carbon source. In the first phase of the fermentation, pyruvate levels rose to 0.09-0.12mg-mL$^{-1}$ at approximately 10h. Following this, the pyruvate concentration decreased rapidly, stabilising at around 0.02mg-mL$^{-1}$ until the end of the fermentation at 50h. No other acidic excretory products were detected or reported in this study.

More recently, it was discovered that the rapid growth of the chloramphenicol producer \textit{S. venezuelae} in minimal medium containing glucose was associated with strong acidification of the medium (Chatterjee & Vining, 1982; Chatterjee et al., 1983). Supplementation of the medium with morpholinopropanesulphonate buffer (MOPS) was required to prevent the pH falling to levels which inhibited growth. The pH of the culture still typically fell from 7.0 to around 5.2 at approximately 48h (corresponding to the maximum biomass yield and the total exhaustion of glucose), then rose to around 6.2 by 120h. This effect was restricted to glucose-grown cultures and was not seen when other carbon sources such as lactose were used. Glucose was preferred to lactose and tended to be exhausted from media by 48h, while only around 75% of lactose was utilised during each 120h culture. It should be noted that MOPS buffer does not always alleviate acidification effects in \textit{Streptomyces}. Zhang et al. (1996) found that MOPS did not prevent the pH of cultures of \textit{S. griseofuscus} in defined glucose-ammonium media falling to growth-inhibitory levels.

Further investigation by Ahmed et al. (1984) revealed that \textit{S. venezuelae} produced high levels of pyruvate and \textit{$\alpha$-ketoglutarate} accompanied by trace amounts of citrate and succinate, generally during nitrogen-limited growth on glucose. Comparison of media containing excess glucose and a selection of nitrogen sources demonstrated that acid levels varied with the nitrogen source used. Acid production was highest following exhaustion of rapidly-used nitrogen sources such as potassium nitrate and ammonium sulphate, both of which were exhausted before 40h during 120h fermentations. The use of the chelating agent magnesium phosphate to trap ammonium ions in an insoluble complex caused increased acidification, indicating that nitrogen-limitation promoted diversion of supplied carbon to excretory products as organic acids.

Both the timing and levels of acid production varied between amino acids tested as nitrogen sources but with no general trend apparent, except that in several cases acid
production occurred while the amino acid was still present at high levels in the medium. Interestingly, subsequent experiments using a mixed nitrogen source in the form of ammonium nitrate showed that pyruvate and α-ketoglutarate were produced when ammonium was exhausted but while nitrate still remained at high levels, indicating differences in the metabolic response to the different nitrogen sources. In the latter experiments glucose exhaustion occurred and was followed by rapid reassimilation of pyruvate and slower reuse of α-ketoglutarate.

The authors then compared the performance of high and low acid-producing strains of *S. venezuelae*. Culture extracts were assayed for activity of the enzymes pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (PDH and α-KDH, complexes which degrade pyruvate and α-ketoglutarate respectively). It was found that neither strain showed any PDH activity, while α-KDH activity varied between the two. α-KDH activity was high in both types during early growth, then in the high-producing strain activity disappeared by 96h and was not resumed. Conversely, in the low-producing strain α-KDH activity increased throughout the culture. Hence, loss of α-KDH activity resulted in increased excretion of acid and the apparent failure of the cells to reassimilate acid after glucose exhaustion, although no differences were detected in the α-ketoglutarate uptake abilities of the two strains. Strain differences were only observed in glucose-limited media. With excess glucose present, both strains were high acid-producers with the pH of the medium dropping as low as 4.5 and inhibiting growth. It was suggested that acid production was due to the regulatory suppression of the dehydrogenase enzymes by glucose, a phenomenon which is known to occur with α-KDH in *Escherichia coli* (Guest, 1992); and that the low-producing strain had reduced sensitivity of α-KDH to glucose. Further examples of the effect of glucose on metabolic pathways and enzymes in *Streptomyces* will be discussed in Section 1.3. Later work conducted using *S. venezuelae* required the use of pH-control in fermenters (Shapiro & Vining, 1983 and 1984).

Surowitz & Pfister (1985) reported that the growth of *S. alboniger* in complex media containing glucose was also accompanied by acid excretion, in this case exclusively pyruvate. Acid excretion interfered with the development of the organism, inhibiting the formation of aerial mycelia. The effect was again found to be restricted to growth on glucose and did not occur when other carbon sources (including dextrin, glycerol and fructose) were used as the sole carbon sources. The acid effect could be
prevented by buffering the medium. Comparison of three types of complex media showed that biomass yields were highest and pH changes were minor in medium containing dextrin. No acids were detected by HPLC in dextrin medium. In medium with glucose, growth was poor and the pH fell from 7.0 to 3.5 by 96h, accompanied by the production of \textit{circa} 1.5\text{mg-mL}^{-1} pyruvate. The addition of 5mM adenine to this medium relieved the effects of acid production. In this case, pyruvate levels reached only 0.2mg-mL^{-1} and the pH fell from 7.0 to only 5.7 over 120h, with a corresponding improvement in growth rate and biomass yield (although these were still lower than in the medium with dextrin). The effect of adenine was thought to occur at the transcriptional level of regulation, since added adenine was not found to affect enzyme activity.

Assays of the activities of several enzymes in \textit{S. alboniger} extracts showed that, in contrast to \textit{S. venezuelae}, pyruvate over-production was not due to lack of degradative enzymes such as PDH, but rather to increased activity of glycolytic enzymes including phosphofructokinase and pyruvate kinase (see Figures 1.1 and 1.2) relative to TCA cycle enzymes such as PDH and citrate synthase. In dextrin-based or adenine-supplemented media, the activities of the two pathways were balanced. No pyruvate was reassimilated by the organism (unlike previous examples), although it appears that none of the cultures experienced total glucose exhaustion.

Dekleva & Strohl (1987) found that anthracycline-producing cultures of \textit{S. peucetius} spontaneously switched to producing acidic metabolites. The excreted acids were found to be mainly pyruvate and $\alpha$-ketoglutarate, with low levels of citrate and lactate. In complex media containing glucose, up to 60% of cultures acidified, resulting in poor biomass yield and a fall in pH to as low as 5.5. During a fermentation using a second complex medium containing glucose, the authors found that in the first 48h, the organism grew well and produced little acid. Between 72h and 96h, the pH fell to around 5.0 as acid concentrations increased rapidly (except lactate, which remained at low levels throughout) to give around 1.1g-L^{-1} total acid by 120h. This was composed of approximately 0.3g-L^{-1} pyruvate, 0.5g-L^{-1} $\alpha$-ketoglutarate, 0.25g-L^{-1} citrate and 0.1g-L^{-1} lactate. It appeared that acid production inhibited anthracycline production, since the neutralisation of pH using sodium hydroxide in acidified cultures restored antibiotic production.
Further experiments conducted using nitrate-defined medium with a selection of primary carbon sources demonstrated that acidification was most common when glucose was the sole carbon source (70% acidified), less common with fructose (40% acidified) and did not occur when maltose, starch or dextrin were used. In acidified cultures, the pH fell as low as 4.7 and biomass yields were lower compared to non-acidified cultures. The authors also noted that *S. peucetius* cultures rarely acidified in complex media without glucose, indicating an effect specific to certain carbon sources, as seen in both *S. venezuelae* and *S. alboniger* (Ahmed *et al.*, 1984; Surowitz & Pfister, 1985). However, *S. peucetius* differed in several respects when compared to other acid-producing *Streptomyces*. First, in all previous examples, acidification was growth-associated with maximum acid excretion occurring between 24 and 48h, whether in defined or complex media. *S. peucetius* cultures tended to acidify after the growth phase had finished, usually after at least 72h. Second, *S. peucetius* did not reassimilate excreted acids, although the authors did not report whether glucose was exhausted in any of the cultures, which appears to trigger acid reuse in other strains. The authors suggested instead that failure to reuse acids was due to the toxic effect of the very low culture pH. Third, assays of the activities of enzymes including PDH and α-KDH showed no significant differences between acid-producing and non-acid-producing cultures. Maltose, which is a dimer of glucose and would be metabolised via much the same pathways, did not induce acid production. This led the authors to suggest that the transport and phosphate-activation mechanisms of glucose metabolism may be involved in acid excretion by *S. peucetius* instead.

The production of organic acids was observed by Hobbs *et al.* (1992) while studying the synthesis of methylenomycin by *S. coelicolor*. During growth in a defined medium (HMM: Hobbs’ minimal medium) containing glucose and alanine, this organism excreted pyruvate and α-ketoglutarate. Acid release occurred while glucose was utilised from the medium, and was accompanied by a fall in pH to *circa* 5.5 at about 66h. Maximum acid levels detected were approximately 0.27mg·mL⁻¹ α-ketoglutarate and 0.22mg·mL⁻¹ pyruvate. After 66h, growth ceased, pyruvate was rapidly reused, α-ketoglutarate levels stabilised and the pH rose to around 7.5 at 120h. In comparison to *S. alboniger* and *S. peucetius* (in which glucose or acid by-products of its metabolism were found to inhibit antibiotic production), methylenomycin synthesis occurred during a transitional period of slow growth between the exponential and stationary phases; which
in turn coincided with the period of maximal glucose consumption and the increase in acid concentrations. It was reported that maximum methylenomycin levels were detected at around the same time as maximum pyruvate and \(\alpha\)-ketoglutarate levels (about 66h), indicating that methylenomycin production was not affected by medium acidification. Indeed, the authors suggested that methylenomycin synthesis may be a stress response by the organism to environmental pH changes. A later report by Obanye et al. (1996) suggested that methylenomycin production coincided with pyruvate reassimilation and that the two were related. Fluctuations in pH during \textit{S. coelicolor} cultures were also observed by Tough & Prosser (1996) during recent morphology studies.

Recombinant strains of \textit{S. lividans} 66 producing the \textit{Flavobacterium} enzyme parathion hydrolase (Steiert et al., 1989; Payne et al., 1990; DelaCruz et al., 1992) have also been found to excrete organic acids. Payne et al. (1990) observed that in shake flask cultures, supplementation of standard Luria-Bertani medium with moderate amounts of glucose (30g-L\(^{-1}\)) improved growth and product yield. However, increasing the initial glucose actually resulted in reduction of both biomass and enzyme levels. Increased glucose led to reduced pH of the culture, in a linear relationship, with the pH dropping to 5.0-5.5 with 53g-L\(^{-1}\) initial glucose.

When tested in a fermenter it was found that a pH-controlled batch culture with 30g-L\(^{-1}\) initial glucose resulted in a slight enhancement of product levels. Feeding of glucose in a fed-batch culture resulted in doubled enzyme yield. Furthermore, feeding of both glucose and tryptone led to a six-fold increase in production. Although no report of pH changes during these fermentations is given, the authors suggested that feeding of substrates resulted in more efficient conversion of the substrates to product, alongside reduction of substrate conversion to organic acids.

At this stage, the authors were unable to identify the acid produced, but did not detect pyruvate or \(\alpha\)-ketoglutarate. In a later report (DelaCruz et al., 1992) it was reported that the acidification of the medium was due to accumulation of isovaleric acid and lesser amounts of acetic acid. During a batch fermentation, isovalerate levels reached a maximum of 2.4mg-mL\(^{-1}\) and acetate reached 0.8mg-mL\(^{-1}\) (compared to maxima of 0.8mg-mL\(^{-1}\) and 0.15mg-mL\(^{-1}\) respectively in a fed-batch culture with fed glucose and tryptone). It was suggested that isovalerate accumulation was due to incomplete metabolism of the peptide (tryptone) component of the medium, since it was
known that isovaleryl CoA is derived from leucine deamination; rather than to the overflow from glucose catabolism seen in previous examples.

A theory was proposed by the authors, whereby under conditions of excess glucose, amino acids are used by the organism as a source of nitrogen. This would result in the accumulation of acidic deamination by-products, such as isovalerate, and a fall in pH. Conversely, when insufficient glucose is available, amino acids are used instead to supply carbon. This would lead to the accumulation of ammonium ions in the medium, causing a rise in pH. The first part of this theory would explain the production of isovalerate and is indirectly supported by Erpicum et al. (1990) These workers noted that recombinant S. lividans TK24 did not suffer pH fluctuations when extra ammonium salts were added to a complex medium (MYEME, containing glucose, sucrose, tryptone, yeast extract, malt extract and buffer) to prevent the catabolism of the amino acid constituent. When ammonium salts and buffer were absent, the pH of the culture fell rapidly to circa 4.5-5.0 and enzyme production was inhibited. No attempt was made during that study to identify the acid(s) produced.

It should be noted that the addition of isovalerate to high-producing cultures of recombinant S. lividans 66 by DelaCruz et al. (1992) did not affect production, indicating that the conditions in which this by-product accumulated may affect production, rather than the acid itself.

During another study (Wrigley-Jones, 1991; Wrigley-Jones et al., 1993) host and recombinant strains of S. lividans TK24 were found to excrete copious amounts of acidic metabolites during rapid growth in a complex medium containing malt extract, peptone and glycerol (MEP). Unchecked acid production reduced the culture pH as low as 2.0-3.0, which had a deleterious effect on growth. Further 7L (5L working volume) fermentations were conducted with pH control. Each required the addition of between 115mL and 140mL of 4M sodium hydroxide to maintain neutrality depending on strain and conditions. These volumes equate to the production of between 0.45 and 0.55 mol equivalents (meq) of organic acid(s) during the course of each 120h fermentation. In these pH-controlled cultures it was observed that acid production coincided with the exponential growth phase and that both ceased simultaneously at about 48h. After this, the pH rose unchecked to reach a final value of 8.5-9.0. It thus appeared that excreted organic acids were reassimilated by the cells, possibly due to exhaustion of the primary
carbon source (glycerol), while the organism remained metabolically active during a period of secondary growth preceding true stationary phase (i.e. the transition phase).

It was suggested that acid production was due to a metabolic imbalance which was possibly caused by rapid glycolysis compared to relatively slow activity of the TCA cycle during the uptake of glycerol. Such an imbalance, together with the associated pH fluctuations, might affect the performance of a secreted active product from a recombinant strain. Analysis of samples showed the organic acid(s) produced to be those other than lactate, citrate, oxaloacetate, malate, succinate or glutamate (unpublished data), but their identity was not determined in that study.

To summarise, the excretion of organic acids into the extracellular medium appears to be a fairly common phenomenon in *Streptomyces*. In general, products are α-keto acids associated with glycolysis, the TCA cycle and occasionally the degradation of amino acids. However, the number and types of acids produced, the timing of their production, and the actual source in the medium (generally a rapidly-used carbon source such as glucose) varies greatly between species.
1.2.2 Acid Production by *Escherichia coli*

The following section describes acid production by *Escherichia coli*. It will show the comparisons between the acidification phenomenon in this well-characterised Gram-negative bacterium and that in streptomycetes.

*E. coli* is found to excrete acetate during aerobic growth on excess glucose and other carbon sources (El-Mansi & Holms, 1989). Acetate is formed from acetyl CoA via the catabolic phosphotransacetylase/acetate kinase pathway (Brown *et al.*, 1977; El-Mansi & Holms, 1989). This reaction is shown in Equation 1.1.

\[
\text{acetyl CoA} + \text{Pi} \xrightarrow{\text{phosphotransacetylase}} \text{acetyl phosphate} + \text{CoA} \\
\text{acetyl phosphate} + \text{ADP} \xrightarrow{\text{acetate kinase}} \text{acetate} + \text{ATP}
\]  

(Equation 1.1)

Acetyl CoA is the end product of glycolysis, converted from pyruvate by the PDH complex, as shown in Figure 1.2. Growth on pyruvate as the sole carbon source is found to result in the highest flux of supplied carbon to acetate. This is understandable since, irrespective of the primary carbon source, all carbon which ends up as acetate passes through pyruvate (El-Mansi & Holms, 1989).

Following exhaustion of the primary carbon source, or at high acetate concentrations, *E. coli* is able to reassimilate acetate. This is thought to occur via two pathways. The first is the conversion of acetate directly to acetyl CoA, catalysed by the inducible enzyme acetyl CoA synthetase, as shown in Equation 1.2.

\[
\text{Acetate} + \text{CoA} + \text{ATP} \xrightarrow{\text{acetyl CoA synthetase}} \text{acetyl CoA} + \text{ADP}
\]  

(Equation 1.2)

However, the maximal rate of acetyl CoA formation by this irreversible pathway is too low for it to account for the ability of *E. coli* to rapidly take up excreted acetate. It is thought likely that this pathway is used to 'scavenge' acetate, particularly when it is present at low concentrations. At high concentrations the reactions catalysed by ACK (acetate kinase) and PTA (phosphotransacetylase) are reversed and these enzymes form
part of a second, anabolic, pathway for the conversion of acetate to acetyl CoA (Brown et al., 1977).

The action of these pathways allows *E. coli* to be grown on acetate as the sole carbon source, although it is a poor substrate when compared to others including glucose, glucose-6-phosphate, fructose, glyceraldehyde, pyruvate, lactate or fumarate (Holms, 1987; El-Mansi & Holms, 1989). It has been reported that when acetate is used by *E. coli* as the sole carbon source, both the TCA cycle and another pathway, the glyoxylate bypass (Figure 1.3) operate.

At the simplest level, carbon flowing through the glyoxylate bypass is used to supply biosynthetic precursors, while carbon flowing through the TCA cycle is used to supply reducing power and ATP, part of which is used to convert precursors to biomass (Holms, 1987; Nimmo et al., 1987). Biosynthesis is limited by the rate of precursor supply (i.e. flux through isocitrate lyase (ICL) and from acetate to acetyl CoA) whilst flux through isocitrate dehydrogenase (ICDH) is adjusted so that the supply of ATP and NADPH are equal to the demand for biosynthesis. In the steady state, the fluxes through ICDH and ICL have a ratio of approximately 2:1. ICDH is restrained by reversible phosphorylation (by a bifunctional kinase/phosphatase). ICL and malate synthase are inducible. It can be surmised that this double system is used to maximise the use of acetate by the organism, since one acetyl CoA unit is used during each turn of the TCA cycle for the generation of energy and precursors, and two acetyl CoA units are metabolised by the glyoxylate bypass.

Acetate is a fermentative metabolite and would be expected as a product from *E. coli* when subjected to oxygen starvation, so why is it excreted under aerobic conditions? There are two main theories explaining why acetate should be in aerobic conditions. The first is supported by El-Mansi & Holms (1989) and others including Konstantinov et al. (1990) and Han et al. (1992). In this theory, acetate is excreted when the input carbon flux exceeds the anabolic capacity of the central metabolic pathways (CMPs). El-Mansi & Holms (1989) explain that excess carbon may be disposed of as storage polymers such as glycogen; as CO₂; or as excreted low-molecular weight compounds such as acetate. In other words, acetate excretion is not due to oxygen deficiency but to metabolic imbalance resulting from saturation of the electron transport chain.
Figure 1.3: Diagram showing the glyoxylate bypass. Solid arrows indicate reactions identical to those in the TCA cycle, dotted arrows indicate reactions specific to the bypass (after Stryer, 1995).
This theory is supported by Konstantinov et al. (1990) who suggested that cells have a limited respiratory capacity. When glucose is in excess, the oxidative capacities are exceeded and carbon is directed through fermentative metabolic pathways, resulting in acetate excretion. The 'overload' theory is extended by Han et al. (1992) who suggested that at high growth rates, anabolic and catabolic requirements exceed the limited capacity of oxidative metabolism (TCA cycle). Here, E. coli metabolism is reorganised so that anabolic needs are met first; then the energy requirements are satisfied by using the remaining oxidative capacity and by the formation of acetate. Acetate synthesis is used as an aerobic energy source since the process generates the second largest amount of ATP and NADH₂ after glucose metabolism. El-Mansi & Holms (1989) also pointed out that the ACK/PTA pathway provides advantages to the cells, by the generation of ATP and CoA for the free intracellular pool (Equation 1.1).

An alternative theory is given by Guest (1992). It is known that the TCA cycle is subject to glucose-mediated repression in E. coli (see Section 1.3 for a discussion of catabolite regulation) in a similar way to the effect of anaerobiosis. Under either anaerobic conditions or aerobic conditions in the presence of excess glucose, the TCA cycle switches to a branched, non-cyclic form. In this situation, a reduced flow of carbon is sustained to α-ketoglutarate and succinate, which satisfies the biosynthetic functions of the cell; while the major part of the carbon flux passes to fermentation products including acetate, ethanol and formate, with low recovery of utilisable energy. It is noted in this article that most of the TCA cycle enzymes are repressed by glucose. The α-KDH complex is severely repressed and the PDH complex is partially repressed and inhibited. Ahmed et al. (1984) also noted that α-KDH is repressed by glucose in E. coli, and suggested a similar effect in S. venezuelae. However, pyruvate and α-ketoglutarate are not produced by E. coli during growth on excess glucose, demonstrating the metabolic differences between these bacteria. In addition, El-Mansi & Holms (1989) have noted that acetate is not excreted during growth on glycerol (for example) when the carbon flux into the CMPs matches the demands for biosynthesis and energy generation. This can be explained by either of the above theories and it remains to be seen which theory is accurate. A version of the 'glucose repression' theory is mentioned by Han et al. (1992) but is rejected by Konstantinov et al. (1990). It remains irrefutable that the flux of excess carbon into E. coli cells results in the excretion of metabolic intermediates.
Acetate excretion by *E. coli* cultures inhibits growth and reduces recombinant protein yield (Han *et al.*, 1992; Kleman & Strohl, 1994; Turner *et al.*, 1994). It may be associated with toxic effects and represents the wasteful conversion of supplied carbon to undesirable by-products due to metabolic imbalance (DelaCruz *et al.*, 1992). Koh *et al.* (1992) reported that acetate added to media had a more significant inhibitory effect on the growth rate of recombinant strains than host strains. Koh *et al.* (1992) also note that the inhibitory effect of acetate on growth was less with all strains tested in complex media (mineral salts base with added glucose and yeast extract or casein hydrosylate) compared to defined media (base medium with glucose and specific amino acids). Han *et al.* (1992) reported that the addition of yeast extract to a mineral salts medium containing glucose reduced acetate excretion. The presence of yeast extract was found to reduce glucose uptake. The authors suggested that yeast extract supplied precursors such as amino acids, thus reducing the anabolic requirements of the cells and hence the need for excess glucose uptake. Furthermore, use of yeast extract alone resulted in the lowest levels of acetate recorded in that study.

Contradictory results have been reported by Kleman & Strohl (1994) where the addition of complex nutrients such as soybean hydrosylate to recombinant *E. coli* cultures resulted in increased acetate accumulation. Thus there is no general advantage to the use of complex versus defined media. Indeed, Han *et al.* (1992 and 1993) also found that the addition of methionine and glycine to glucose-based minimal media relieved the inhibitory effects of acetate, possibly related to the observed enhancement in cellular respiratory activity. Growth rate and recombinant protein production improved, even while high concentrations of acetate were present.

El-Mansi & Holms (1989) showed that the use of a PDH inhibitor (3-bromopyruvate) prevented flux from the carbon source pyruvate to acetate and allowed improved efficiency of carbon conversion to biomass. Also, mutants lacking PTA and ACK did not excrete acetate when grown on pyruvate, although excessive carbon uptake was balanced by lactate excretion in this case. Further experiments conducted by these workers and by Konstantinov *et al.* (1990) demonstrated that restriction of carbon supply/glucose uptake by continuous culture or nutrient feeding could be used to prevent acetate formation by preventing overloading of the cellular metabolic capacity. In both cases a balance was achieved between carbon input and the requirements of biosynthesis and energy generation, improving biomass and product yields considerably.
Turner et al. (1994) also reported the use of carbon source supply restriction to reduce carbon overflow to acetate. An on-line monitoring and control system was employed to measure concentrations of the carbon source (galactose) and the by-product (acetate) in fed-batch cultures. A build up of either analyte resulted in reduction of the galactose feed rate so that the organism could utilise the excess carbon sources and not suffer excessive acetate levels.

In conclusion, acetate accumulation is commonly associated with the rapid aerobic growth of *E. coli* on a carbon source which is present in excess. In contrast to acid excretion observed in streptomyces, the by-product is a fermentative metabolite rather than an intermediate of oxidative metabolism. In both cases, the ability exists to reassimilate the excreted acids. *E. coli* is able to reuse acetate to support growth via the dual action of the TCA cycle and the glyoxylate bypass. Many *Streptomyces* are able to reassimilate exogenous pyruvate and α-ketoglutarate, although the pathways of uptake and the fate of the reused acids remain unclear. Acid production is not desirable to either type of bacterium, since evidence exists that it may interfere with growth and product yield by host and recombinant strains. However, it appears that by-product accumulation is less severe in *Streptomyces* than in *E. coli*. The results of DelaCruz et al. (1992) indicate that acidification and reduced performance of a strain of *S. lividans* 66 only occurred when glucose levels were higher than 30g·L\(^{-1}\). In contrast, Kleman & Strohl (1994) note than a glucose concentration as low as 0.25-0.5g·L\(^{-1}\) may result in the production of large amounts of acetate by *E. coli*.

*Streptomyces* species are popular for large-scale industrial/commercial production of products including enzymes and antibiotics (Piret & Demain, 1988; Gusek & Kinsella, 1992). There are few published examples of the use of *S. lividans* in large-scale applications, although it is a very popular host for gene cloning and expression of recombinant products at laboratory and small fermenter scales (Sections 1.1.1.2 and 1.1.2.1). There are a few examples of groups attempting to produce elevated levels of commercially important products in *S. lividans* TK24, presumably with a view to industrial-scale application, in which case events such as acid excretion might be undesirable. These include McKillop et al. (1989) at Apcel/Celltech who reported the cloning of α-amylase which is used in the food and brewing industries; or Koller &

It has been noted recently that *S. lividans* so far has only the potential to be a serious alternative to *E. coli* and *S. cerevisiae* for industrial production of recombinant materials (Kleman & Strohl, 1994; Payne *et al.*, 1990). It is also noted that levels of expression of recombinant products need to be improved before *S. lividans* can have economically viable large-scale usage. It has been described previously in this section how Payne *et al.* (1990) demonstrated that the feeding of carbon and nitrogen sources improved recombinant product yield and stability by reducing excretion of acidic by-products. DelaCruz *et al.* (1992) note that many industrial fermentations are run in a fed-batch format to avoid such by-product accumulation.
1.3 Streptomyces Secondary Metabolism and Catabolite Regulation

1.3.1 The Control of Streptomyces Metabolism

Metabolic changes in streptomycetes are subject to a number of control mechanisms of which probably the best documented is the phenomenon of catabolite regulation. Carbon, nitrogen and phosphate sources can affect essential enzymes and the synthesis of secondary metabolites. It is possible that these sources may affect organic acid production and pigmentation by *S. lividans*. This section summarises the control of metabolic changes and the following sections will deal with carbon regulation (1.3.2), nitrogen regulation (1.3.3), phosphate control (1.3.4) and interactions of these (1.3.5).

The *Streptomyces* life cycle generally consists of two stages, a vegetative growth phase (also called 'trophophase') and a production phase (also called 'idiophase'). Secondary metabolites, including antibiotics, pigments and biologically active compounds (perhaps also encompassing organic acids; Liras *et al.*, 1990) tend to be produced in the latter stage (Chater, 1989; Demain, 1992). However, as defined by Demain, secondary metabolites are not considered secondary because they are produced after growth, but because they are not essential for growth (Martin & Demain, 1980; Piret & Demain, 1988; Demain, 1992).

The growth and production phases may not be distinct in *Streptomyces*. Dry cell weight is often used as an indicator of growth but biomass may continue to accumulate at a significant rate into the production phase. This is due to the accumulation of storage materials and not true replicatory growth. Other parameters, including deoxyribonucleic acid synthesis and respiratory activity, give a more accurate indication of the end of the growth phase (Martin & Demain, 1980).

It has also been observed that the phase in which secondary metabolism occurs is dependent on the nutritional environment. In batch cultures using complex media supporting rapid growth, growth is typically biphasic. Distinct growth and production stages are seen and secondary metabolism is growth-dissociated. In defined media supporting slower growth, the phases overlap and secondary metabolism is growth-linked (Martin & Demain, 1980; Piret & Demain, 1988; Wallace *et al.*, 1992; Demain, 1992). It is thought that secondary metabolism is initiated by nutrient limitation and the end of catabolite regulation by carbon, nitrogen or phosphate sources. In complex
media, production occurs when a key nutrient is depleted and the growth rate decreases; in defined media key nutrients may be limiting from the start of the culture, causing simultaneous growth and production (Martin & Demain, 1980; Wallace et al., 1992; Liao et al., 1995). Wallace et al. (1992) reported that the production of streptonigrin by *S. flocculus* was clearly suppressed during rapid growth in complex medium. In defined medium, the growth rate was slower and production was growth-associated. In contrast, Hobbs et al. (1990) observed that production of actinorhodin by *S. coelicolor* occurred in the stationary phase in a defined medium, while undecylprodigiosin was produced in the rapid growth phase. The products were affected to different degrees by the nitrogen and phosphate sources. However, Liao et al. (1995) reported that actinorhodin production was dependent on a slow growth rate occurring either in the stationary phase, or throughout cultures in nutrient-limited media. These results indicate that generalisations do not always hold true between *Streptomyces* strains and products.

Since secondary metabolism generally occurs at low growth rates, it is also possible that growth rate alone may be a control factor. Secondary product synthesis is also subject to further regulation by enzyme induction and inactivation and product feedback inhibition. The control factors are complex and poorly understood (Martin & Demain, 1980; Demain, 1992). Regulatory mechanisms have been reviewed recently by Bibb (1996). Only growth rate and carbon, nitrogen and phosphate source effects will be considered here, although their roles as signals for morphological and biochemical differentiation remain to be proved (Bibb, 1996).

Overlapping growth and production phases may be caused by other factors which result in reduced growth rate and nutrient limitation. Many *Streptomyces* strains form mycelial pellets, particularly in defined media (Section 1.4). The interior biomass may become nutrient limitation and switch to secondary metabolism while the peripheral biomass continues to grow rapidly. Secondary product formation may also be initiated by the use of inocula already engaged in secondary metabolism; or by the decrease in growth rate caused by transferring pregrown inoculum from complex to defined media (Doull & Vining, 1989 and 1990a; Liao et al., 1995). Supplying carbon, nitrogen or phosphate in a form not readily utilised by the organism also simulates nutrient-limited conditions (Chatterjee et al., 1983).
Readily-utilisable carbon, nitrogen and phosphate sources supporting rapid growth may act by repressing the synthesis of certain enzymes at the transcriptional level or, less frequently, by inhibiting their activity (Martin & Demain, 1980; Piret & Demain, 1988; Demain, 1992). These effects have been embraced by the term 'catabolite regulation' (Doull & Vining, 1990a). A preferred carbon source can suppress enzymes of pathways involved in the metabolism of alternative carbon sources. Carbon, nitrogen and phosphate sources can interfere with secondary metabolic pathways. It is thought that carbon, nitrogen and phosphate-regulation mechanisms evolved to restrain unnecessary activities such as secondary metabolism when rapid growth was possible (Doull & Vining, 1990a). Alternatively, this may be a mechanism to delay antibiotic production until it is required for defence of the colony at differentiation (Demain, 1992). Intriguingly, it is also suspected that high levels of the phosphorylated nucleotide ppGpp are related to antibiotic production under certain nutritional conditions such as amino acid starvation (Bibb, 1996).

When the suppressive nutrient becomes limiting, the enzymes are derepressed and the uptake of alternative sources and/or secondary metabolite synthesis can occur. Catabolite regulation is discussed in the following sections and a recent review of examples is given by Demain & Fang (1995).
1.3.2 Carbon Regulation

Readily assimilated carbon sources may interfere with metabolic pathways. Glucose is a common example and is found to suppress both antibiotic production and the assimilation and metabolism of other carbon sources (Doull & Vining, 1990a).

1.3.2.1 Antibiotics

To promote antibiotic production it is better to use polysaccharides, oligosaccharides or oils rather than glucose. There are specific alternative non-interfering carbon sources for many antibiotics (Demain, 1992). In media containing glucose and a slowly-used carbon source, a diauxic growth pattern may be seen. Glucose is used exclusively during the early part of the culture to support rapid growth in the absence of production. On glucose exhaustion, the second carbon source is used and antibiotics are produced. These alternatives to glucose include glycerol, galactose, sucrose and starch, although these vary between strains (Martin & Demain, 1980).

As an example, Gallo & Katz (1972) reported that actinomycin synthesis by *S. antibioticus* was suppressed by glucose and other rapidly-utilised carbon sources such as lactose and mannose. Maltose and sucrose were less repressive. Maximum antibiotic production was obtained in a defined medium containing 0.1% glucose and 1% galactose. Glucose was used first for rapid growth and galactose later for slow growth and antibiotic production. These results may suggest a dependence on growth rate, since maltose is a dimer of glucose and would be metabolised *via* the same pathways, but is one of the least repressive. However, it was found that glucose specifically repressed the formation of the biosynthetic enzyme phenoxazinone synthase (PAS). Recent work (cited by Doull & Vining, 1990a and Demain, 1992) demonstrated that glucose repression acted at the transcriptional level. PAS transcripts were detected at lower levels during the growth phase than the production phase in the glucose-galactose medium, and at lower levels still in medium containing only glucose.

Wallace *et al.* (1992) suggested that another solution would be to limit the availability of glucose by feeding, although Martin & Demain (1980) noted that this may just indicate growth rate control effects. However, the use of the glucose analogue methyl-α-glucoside reduced glucose uptake and increased the yield of chloramphenicol by *S. venezuelae* (cited by Wallace *et al.*, 1992).
Glucose is not always a repressive carbon source (Demain, 1992). Aharonowitz & Demain (1978) tested a number of carbon sources, combined with asparagine as the nitrogen source and secondary carbon source, on growth and cephalosporin production by *S. clavuligerus*. Growth on glycerol, maltose and starch gave the highest cephalosporin titre. Glycerol and maltose supported with high biomass but low antibiotic yield. Starch supported slower growth and higher antibiotic yield. Since production was growth-associated on starch, it was suggested that starch was a limiting carbon source which relieved carbon repression. The organism would not grow on glucose. Later work by Lebrihi *et al.* (1988) showed that glycerol and starch repressed the formation of the biosynthetic enzyme ‘expandase’ (deacetoxycephalosporin C synthetase) at the transcriptional level. Glycerol was more repressive than starch, causing a 40% reduction in the levels of expandase detected.

The expression of morphological development genes (*e.g.* bld and whi genes) is affected by the carbon source in *S. coelicolor* (Ingram *et al.*, 1995). Carbon source-specific mutants defective in both differentiation and antibiotic production have been identified (reviewed by Piret & Demain, 1988). In general, abnormal growth is seen in mutants grown on glucose or cellobiose, while mutants exhibit wild type differentiation when grown on maltose, glycerol or galactose. There are examples in other strains, for example glucose represses both sporulation (Surowitz & Pfister, 1985) and puromycin production (cited in Doull & Vining, 1990a) by *S. alboniger*. This indicates complex control mechanisms in this genus.

**1.3.2.2 Assimilatory Enzymes**

The possibility that glucose may repress essential primary metabolic enzymes such as α-KDH in *Streptomyces* has been mentioned in Section 1.2 (Ahmed *et al.*, 1984). Glucose also interferes with enzymes involved in the uptake and metabolism of other carbon sources in several *Streptomyces* species. The following examples are also reviewed by Doull & Vining (1990a).

Chatterjee & Vining (1982) reported that β-galactosidase, which mediates lactose assimilation in *S. venezuelae*, was induced by lactose and repressed strongly by
glucose, maltose or acetate and weakly by glycerol and citrate. Growth in glucose-lactose media was diauxic, first on glucose and later on lactose.

It has been reported that several α-amylases, which permit growth on starch, are suppressed by alternative carbon sources. Long et al. (1987) noted that the expression of α-amylase by *S. limosus* is induced by maltose (a degradation product of starch) and repressed by mannitol in the parent strain or by glucose in recombinant *S. lividans* TK24. Subsequently, Virolle et al. (1988) found that the *S. venezuelae* α-amylase gene was induced by maltose and repressed by glucose in both the wild type strain and in recombinant *S. lividans* TK24 or *S. coelicolor*. The cloned α-amylase genes were found to contain identical promoters. The authors surmised that the insensitivity of *S. limosus* to glucose was a characteristic of the strain rather than the α-amylase gene itself. Recently, Virolle & Gagnat (1994) showed that deletions from the promoter region of the *S. limosus* α-amylase gene render it resistant to glucose repression and insensitive to maltose induction, indicating complex control mechanisms at the transcriptional level.

Hodgson (1982) discovered that *S. coelicolor* agarase was also suppressed by glucose. Servin-Gonzalez et al. (1994) reported that the agarase gene, *dagA*, was induced by agar degradation products and repressed by glucose at the level of transcription. Transcripts were found to be at low levels in media containing glucose. When *dagA* was cloned into *S. lividans* TK24 (which lacks agarase) on a low copy number plasmid, it was again repressed by glucose. In contrast, when the gene was cloned into *S. lividans* TK24 on a multicopy vector glucose repression was not as severe, presumably due to the high number of *dagA* genes present (cited in Gusek & Kinsella, 1992).

In *S. coelicolor*, the utilisation of other carbon sources (including glycerol, fructose, galactose and arabinose) is affected by glucose (Hodgson, 1982). This work will be discussed in more detail in Section 1.3.2.3 in relation to carbon regulation mechanisms.

### 1.3.2.3 Mechanisms of Carbon Regulation

While glucose and other carbon sources undoubtedly regulate the expression and activity of certain enzymes, still little is known about the mechanism of carbon catabolite regulation in *Streptomyces* (Ingram et al., 1995).
In *E. coli*, the glucose repression effect [after which catabolite repression/regulation in streptomycetes was named (Doull & Vining, 1990a)] is mediated by cyclic AMP (cAMP). At high glucose concentrations, the cAMP synthetic enzyme adenylate cyclase is deactivated via the phosphoenolpyruvate-phosphotransferase carbohydrate transport system (PTS or PCT) engaged in glucose uptake. This reduces the intracellular cAMP concentration. At low glucose concentrations, cAMP levels rise and cAMP binds to the cAMP receptor protein (CRP). This complex binds to the promoter sites of repressed, inducible genes (e.g. the β-galactosidase lac operon) and activates transcription (Martin & Demain, 1980; Doull & Vining, 1990a; Stryer, 1995).

In contrast, cAMP does not seem to have a regulatory role in carbon regulation in *Streptomyces* (Demain & Fang, 1995). In *S. venezuelae*, cAMP levels were not found to change significantly at glucose exhaustion or the onset of lactose utilisation/β-galactosidase derepression and chloramphenicol production (Chatterjee & Vining, 1982). Similarly, cAMP levels remained constant at glucose depletion and the assimilation of alternative sources by *S. coelicolor* (Hodgson, 1982). Other examples where variations in cAMP could not be correlated to physiological changes are reviewed by Doull & Vining (1990a). These authors, with Angell *et al.* (1992), Servin-Gonzalez *et al.* (1994) and Ingram *et al.* (1995), note that various streptomycetes, including *S. coelicolor*, lack an equivalent PTS system for sugar uptake. Instead, glucose is phosphorylated directly after assimilation by the constitutively expressed enzyme glucose kinase (GLK).

It is possible that carbon repression in *S. coelicolor* and other streptomycetes may involve GLK. In 1982, Hodgson showed that glucose interfered with the utilisation of several other carbon sources by *S. coelicolor*. Glucose repressed or inhibited enzymes involved in the substrate-inducible uptake of glycerol and arabinose and in the uptake and metabolism of galactose and fructose. Hodgson isolated mutants which were pleiotropically derepressed and grew well on at least 10 other carbon sources (including agar) in the presence of the glucose analogue 2-deoxyglucose (DOG). DOG is phosphorylated and, although not metabolised to completion, does initiate glucose repression, hence the mutants were called Dog*. Some mutants were unable to grow on glucose or its polymers (maltose, starch, cellobiose), although the constitutive
assimilation mechanism was not affected. From further results, Hodgson concluded that Dog\textsuperscript{R} mutants lacked glucose kinase.

This was confirmed by Seno & Chater (1983), who noted that Dog\textsuperscript{R} mutants which lacked glucose repression of enzymes involved in glycerol utilisation also lacked or contained only low levels of GLK. These authors suggested that Dog\textsuperscript{R} mutants arose due to mutations in the glucose kinase gene (glk). Subsequently, Ikeda \textit{et al.} (1984) reported that the cloning of a DNA fragment containing glk from \textit{S. coelicolor} into Dog\textsuperscript{R} mutants restored GLK activity, the ability to utilise glucose, DOG sensitivity and glucose repression effects. They suggested that GLK may act indirectly via the formation of a regulatory metabolite, or that GLK may have a direct regulatory function. Angell \textit{et al.} (1992) found that a protein encoded by the glk fragment showed significant homology to members of a family of regulatory proteins from bacteria including \textit{E. coli} and \textit{Bacillus subtilis}. However, the absence of DNA-binding regions in the product suggested it was unlikely to be a direct transcriptional regulator.

Angell \textit{et al.} (1992) and Servin-Gonzalez \textit{et al.} (1994) list a number of enzymes repressed at the transcriptional level by glucose, and which are relieved in glk mutants. These include \textit{dagA}, \textit{aml} (Long \textit{et al.}, 1987; Virolle \textit{et al.}, 1988) and the glycerol utilisation operon \textit{gyl} (Hodgson, 1982; Seno & Chater, 1983). In 1995, Ingram \textit{et al.} reported the identification of a mutation, \textit{ccrA1}, which pleiotropically affected the expression of several catabolite regulated promoters. It seemed that \textit{ccrA1} may be part of a general catabolite control mechanism in \textit{S. coelicolor}. However, certain genes were still repressed by glucose in \textit{ccrA1} mutants and certain genes affected by \textit{ccrA1} were found to be independent of GLK activity. It thus appears that there is more than one mechanism of carbon regulation in \textit{S. coelicolor} (in which secondary metabolism is not affected by the carbon source (Hodgson, 1982)); and in \textit{Streptomyces} species where carbon sources not metabolised \textit{via} GLK are repressive. The possible relationship of carbon regulation and acid over-production by \textit{S. lividans} will be discussed in Section 4.
1.3.3 Nitrogen Regulation

The effect of rapidly-utilised nitrogen sources on secondary metabolic pathways and enzymes involved in the utilisation of alternative nitrogen sources by streptomycetes is less well defined than carbon regulation. There are several examples but few reports on the mechanism and many of these are contradictory (Bascaran et al., 1989a).

1.3.3.1 Antibiotics

Nitrogen sources which are preferred for rapid growth, such as ammonium ions and rapidly-used amino acids, often interfere with secondary metabolism (Demain, 1992). In complex media, it is better to use nitrogen sources such as soybean meal and corn steep solids to promote antibiotic production. These complex nitrogen sources are broken down slowly by the organism, preventing the accumulation of repressive ammonium ions and amino acids. In defined media a poorly metabolised amino acid such as proline can prevent repressive effects (Piret & Demain, 1988; Demain, 1992). The addition of ammonium-trapping agents reduces soluble ammonium concentrations and can prevent repressive effects (cited by Piret & Demain, 1988). Demain (1992) lists a number of examples of repressive and non-repressive nitrogen sources.

Chatterjee et al (1983) and Shapiro & Vining (1983) reported that chloramphenicol production by S. venezuelae was lowest when grown on nitrogen sources supporting rapid growth. Highest yields were obtained by the use of poorly used amino acids including proline and isoleucine, which increased the yield up to 4.5-fold compared to cultures grown on ammonium or nitrate salts.

An earlier example was reported by Aharonowitz & Demain (1979). These authors found that ammonium ions supported the growth of S. clavuligerus, but suppressed both sporulation and cephalosporin production. Nitrate supported neither growth nor antibiotic production. Antibiotic production was growth-associated when ammonium was absent, but in ammonium presence production was delayed until after this nitrogen source was exhausted. Ammonium only depressed antibiotic synthesis if added before production had begun, suggesting control at the level of enzyme formation. Of the amino acids which supported growth and sporulation, asparagine, glutamine (and arginine) were found to support the highest antibiotic titres.
Branca et al. (1985) showed that ammonium ions repressed the formation of the *S. clavuligerus* biosynthetic enzymes cyclase (isopenicillin N synthetase) and to a lesser extent expandase (deacetoxy-cephalosporin C synthase, also affected by the carbon sources; Lebrihi et al., 1988). In both cases enzyme activity was low during early growth on ammonium and increased during the culture. Enzyme activity was lower in ammonium-grown cultures than in those containing asparagine as the nitrogen source. As found by Aharonowitz & Demain, asparagine and glutamine were among the nitrogen sources supporting the highest cephalosporin titre and enzyme activity. Interestingly, proline supported both the lowest production and enzyme activity (Branca et al., 1986). Fang & Demain (1995) reported that ammonium repressed another biosynthetic enzyme called ACVS and induced alanine dehydrogenase, increasing intracellular alanine concentrations which in turn inhibited the activity of the three biosynthetic enzymes above. The addition of magnesium phosphate \([\text{Mg}_3(\text{PO}_4)_2\cdot 8\text{H}_2\text{O}]\) increased antibiotic production by ammonium-containing cultures but not asparagine-based cultures. Lounes et al. (1995) report the use of another chelating agent, calcium phosphate \([\text{Ca}_3(\text{PO}_4)_2]\) to restrict ammonium availability and increase spiramycin production by *S. ambofaciens* 6-fold. Wallace et al. (1992) also report that the addition of ammonium-trapping agents increased the production of streptonigrin by *S. flocculus*.

1.3.3.2 Assimilatory Enzymes

Aharonowitz & Demain (1979) and Martin & Demain (1980) note that ammonium regulates enzymes involved in the assimilation and utilisation of sources which supply cells with available nitrogen (most often as ammonium ions or glutamate). Among the regulated enzymes are histidase, arginase, asparaginase, glutamine synthetase, glutamate dehydrogenase and nitrate and nitrite reductases.

Many of the examples in the literature are contradictory. Shapiro & Vining (1984) found that both ammonium and nitrate ions suppressed chloramphenicol formation by *S. venezuelae*. In cultures with ammonium nitrate as the nitrogen source, the ammonium and nitrate ions were used consecutively and most antibiotic synthesis occurred after both were depleted. Although nitrate utilisation appeared to be repressed by the ammonium ions, the enzyme nitrate reductase was constitutively expressed and did not change at ammonium depletion. Since the addition of ammonium to nitrate-
grown cultures repressed nitrate utilisation, the authors suggested that ammonium ions affected uptake mechanisms rather than metabolic enzymes.

Bascaran et al. (1989a and 1989b) observed that some nitrogen source assimilation enzymes in *S. clavuligerus* (e.g. proline dehydrogenase) were not affected by ammonium. The same was seen by Shapiro & Vining (1984) in *S. venezuelae*, where proline utilisation was not affected during the period of ammonium utilisation. Bascaran et al. (1989a) also discovered that certain *S. clavuligerus* enzymes were repressed by ammonium at the level of enzyme formation, including glutamine synthetase (GS), arginase and urease. In contrast, Shapiro & Vining (1983) found that GS from *S. venezuelae* was not affected by ammonium, while other ammonium assimilation enzymes were, including glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). Bascaran et al. (1989a and 1989b) also note that in *S. coelicolor*, GS and urease are affected by ammonium, while arginase and nitrate reductase are not. Finally, Bascaran et al. (1989a and 1989b) isolated mutants of *S. clavuligerus* showing simultaneous ammoniumderepression of a number of assimilatory enzymes, including GS, arginase and urease. These varied results indicate that nitrogen control does occur in *Streptomyces* but that variations occur between both strains and pathways. Regulatory mechanisms are discussed in Section 1.3.3.3.

### 1.3.3.3 Mechanisms of Nitrogen Regulation

Demain (1992) stated that little is known about the means by which ammonium and amino acids regulate metabolism. It is known that in enteric bacteria such as *E. coli*, nitrogen regulation involves GS, which catalyses the formation of glutamine from glutamate and ammonia (Martin & Demain, 1980). Aharonowitz & Demain (1979) noted that the enzymes which supply *Streptomyces* with nitrogen from nitrogenous compounds (including GS) are regulated by ammonium, but that the regulation of secondary metabolism is a quite different process. Many researchers have studied ammonium assimilation enzymes for clues to a mechanism or link between ammonium ions and secondary metabolism in streptomycetes. In both *Streptomyces* and *E. coli*, ammonium assimilation at low concentrations occurs via the high affinity GS-GOGAT system (Wallace et al., 1992). Attempts to relate the ammonium effect to these enzymes have mostly been unsuccessful (Piret & Demain, 1988; Doull & Vining, 1990a).
Shapiro & Vining (1983) found that GS and alanine dehydrogenase (thought to be involved in ammonium uptake via the reductive amination of pyruvate to alanine) were unaffected by nitrogen source depletion in ammonium, proline or isoleucine-containing cultures of *S. venezuelae*. GOGAT was affected, exhibiting high activity during ammonium assimilation and low activity after ammonium depletion or in the presence of alternative nitrogen sources. GDH (involved in the uptake of ammonium at higher concentrations) was also affected although it was never detected at high levels. The authors suggested that these enzymes were involved in nitrogen regulation; but in 1984 they were unable to correlate changes in chloramphenicol synthesis or nitrate uptake to the extracellular ammonium concentration.

Conflicting results were obtained by Brana et al. (1986), who were also unable to relate GS, GOGAT and alanine dehydrogenase levels to antibiotic production by *S. clavuligerus*. GOGAT was unaffected by the nitrogen source in this species, while GS was repressed and alanine dehydrogenase activity was stimulated by the presence of ammonium. GDH was not detected. It was thought unlikely that alanine dehydrogenase was directly involved in nitrogen regulation. GS activity varied with the nitrogen source; the highest activity was detected with glutamine and asparagine as expected, but also with proline and threonine which supported the lowest antibiotic production.

It was noted by Brana et al. (1986) that none of the *S. clavuligerus* mutants defective in individual ammonium assimilation enzymes (*e.g.* defective in GS) were derepressed for antibiotic production. Bascaran et al. (1989b) isolated *S. clavuligerus* mutants with simultaneous deregulation of several nitrogen assimilation enzymes, indicating some kind of global control mechanism, but again none were derepressed for antibiotic production. The authors noted (1989a) that even in enteric bacteria containing a nitrogen control system linked to ammonium assimilation (*ntr*), regulation varies between metabolic pathways. The authors also mention that *ntr* mutants of *S. lividans* 66 exist, which are defective in nitrogen assimilation which may be related to nitrogen control. Although no evidence exists for an equivalent global regulatory network in Gram-positive bacteria (1989b), *Streptomyces* are likely to exhibit such variations if a nitrogen regulation network does exist. Unfortunately very little new research has been reported in this area. Lounes et al. (1995) reported that ammonium affected spiramycin synthesis by *S. ambofaciens* and that certain proteins were absent from cultures.
subjected to an ammonium pulse, but the proteins or actual mechanism have not yet
been identified.

To summarise, nitrogen sources such as ammonium ions affect various nitrogen
assimilation pathways and the synthesis of secondary metabolites, but no clear single
mechanism has been identified. It remains to be seen whether enzymes of ammonium
assimilation are linked to nitrogen regulation in the same way that glucose kinase
appears to be linked to carbon regulation.
1.3.4 Phosphate Control

Phosphate is also known to affect secondary metabolite formation. To promote antibiotic synthesis, fermentations are usually conducted at phosphate concentrations which are growth-limiting (Martin & Demain, 1980; Demain, 1992). A number of antibiotics whose synthesis requires phosphate-limitation are listed in Demain (1992).

Aharonowitz & Demain (1977) used phosphate at a concentration of 75 to 100mM to buffer pH fluctuations during cultures of *S. clavuligerus*. At a phosphate concentration of 10 to 25mM, the extracellular pH fell from 6.7 to 5.0 by 120h (although no attempt was made to identify the cause of this apparent acidification). Increased phosphate levels also suppressed cephalosporin production. With the use of an alternative organic buffer (MOPS) to stabilise pH, the authors found that 25mM phosphate was the optimum concentration for both a reasonable level of growth and maximum cephalosporin yield. The phosphate control effect in this strain was not discussed, although Lebrihi *et al.* (1988) found that phosphorylated intermediates of glycolysis inhibited the activity of the enzyme expandase, which is also affected by certain carbon and nitrogen sources.

The effect of phosphate varies between strains and products. While 50mM phosphate inhibited 50% of cephalosporin production by *S. clavuligerus*, just 7mM phosphate inhibited 50% of candicidin production by *S. griseus* (Aharonowitz & Demain, 1977). In *S. coelicolor*, actinorhodin production was completely inhibited by 24mM phosphate, but undecylprodigiosin synthesis was not prevented at this concentration (Hobbs *et al.*, 1990).

Phosphate affects a wide range of pathways and there may be several control mechanisms (Martin & Demain, 1980). Phosphate can specifically repress or inhibit phosphatase enzymes involved in biosynthetic pathways containing phosphorylated intermediates (Piret & Demain, 1988; Demain, 1992). Phosphate may also interfere with pathways which do not have phosphorylated intermediates (Demain, 1992). Here, phosphorylated nucleotides such as ATP may act as intracellular effectors (Piret & Demain, 1988). Martin & Demain (1980) noted that the addition of 10mM phosphate to candicidin-producing cultures of *S. griseus* caused an increase in ATP levels, followed by suppression of antibiotic synthesis. In addition, measurement of ATP levels in *S. griseus* extracts showed a fall in concentration before the onset of production. Doull &
Vining (1990a) warn that a decrease in ATP levels may be due simply to changes in energy metabolism resulting from phosphate deprivation, rather than a signal for the initiation of secondary metabolism. However, Piret & Demain (1988) note that cellular ATP content is higher in some ancestral low-producing *Streptomyces* strains than in high-producing mutants.

Again, little is known about the specific regulatory mechanism, but it is possible that phosphate acts at the transcriptional level on certain candicidin biosynthetic enzymes in *S. griseus* (Demain, 1992). Liras *et al.* (1990) found that just 0.1M phosphate halved the formation of the enzyme *p*-aminobenzoic acid synthase (PABAS), controlled at the transcriptional level. Analysis of the gene revealed a phosphate control sequence with over 66% homology to known phosphate control sequences in *E. coli*. Further analysis of *Streptomyces* DNA sequences revealed similar phosphate control sequences in a gene of unknown function in *S. lividans* and other streptomycete genes. The mechanism by which phosphate acts is still not clear, but the authors suggested that an intracellular effector such as ATP may act with a DNA-binding protein to mediate phosphate control. Interestingly, Demain (1992) notes that *afsR* (Section 1.1) appears to specify a phosphorylation-dependent regulator, so it is likely that phosphate affects pigmentation by *S. coelicolor* and *S. lividans*. 
1.3.5 Multiple Interactions and Manipulating Secondary Metabolism

According to Doull & Vining (1990a), most secondary metabolic pathways affected by phosphate are also regulated by the carbon and nitrogen sources. In such interactively regulated systems, carbon, nitrogen and/or phosphate depletion cause a decrease in the growth rate, which may itself have a role in the switch between primary and secondary metabolism (Demain, 1992). Indeed, Piret & Demain (1988) speculate that since secondary metabolite synthesis seems to be dependent on low growth rates in most systems, then maybe carbon, nitrogen and phosphate-limitation merely act to lower the growth rate and that perhaps growth rate alone is the controlling factor.

There are several examples where secondary metabolite synthesis is dictated by a number of factors. As discussed in Sections 1.3.2, 1.3.3 and 1.3.4, cephalosporin production by *S. clavuligerus* is affected by carbon, nitrogen and phosphate sources (Aharonowitz & Demain, 1977, 1978, 1979). Maximum production required a growth-limiting carbon source such as starch; a poorly-used nitrogen source such as asparagine; and a reduced level of phosphate. Fang & Demain (1995) demonstrated that ‘regulation reversal’ could occur under environmental modification such as oxygen restriction, such that ammonium and phosphate stimulated β-lactam production by *S. clavuligerus*.

Chatterjee *et al.* (1983) showed that chloramphenicol production by *S. venezuelae* was affected by carbon and nitrogen sources as well as the growth rate. Chloramphenicol production was found to be a growth-associated process and occurred in the presence of rapidly-utilised glucose and ammonium ions in defined media. Poor production accompanied fast growth, hence yields were increased by the use of growth-limiting carbon and nitrogen sources. Growth on poorer carbon sources (lactose, galactose, starch, cellobiose) increased the yield 1.7 to 2.4-fold compared to glucose. Amino acid nitrogen sources (proline, isoleucine) improved the yield 3 to 4.5-fold compared to ammonium and 2 to 3-fold compared to nitrate ions. Higher yields were obtained by restricting nitrogen availability than by restricting carbon availability.

In a single culture, different products may respond by differing degrees to carbon, nitrogen and phosphate sources and growth rate. This lends itself to the possibility of manipulating or directing product synthesis. In an example cited by Demain (1992), Lilley and co-workers found in 1981 that production of antibiotics by *S. cattleya* required both reduced growth rate and nutrient deficiency. Thienamycin
synthesis required a low growth rate specifically due to phosphate-limitation; while it appeared that cephamycin formation occurred at reduced growth rates caused by any of carbon, nitrogen or phosphate depletion. Further work by Bushell & Fryday (1983) clarified these observations. It was demonstrated that the synthesis of at least three secondary metabolites by *S. cattleya* was initiated by specific nutrient deficiency. In a culture grown in defined medium containing glucose, ammonium ions and phosphate, product formation occurred after the peak in biomass at 33h. Melanin production began at 55h, cephamycin production at 70h and thienamycin production at 115h, corresponding to the depletion of glucose, ammonium and phosphate respectively. It was thought possible to direct secondary product synthesis and obtain sequential product formation.

Actinorhodin production by *S. coelicolor* is also found to be initiated by a reduction in growth rate and is sensitive to the nitrogen and phosphate sources (Doull & Vining, 1990b). It is not affected by the carbon source, beyond the availability of acetate precursors (Hodgson, 1982). Doull & Vining (1990b) grew *S. coelicolor* in a defined medium containing starch, glutamate and phosphate. Increasing the nitrogen and phosphate concentrations above growth-limiting levels both delayed the initiation of and reduced the rate of actinorhodin production. The nitrogen source had a greater effect than the phosphate source. The specific role of each was unclear since actinorhodin was still produced when both sources were present in excess as long as the growth rate decreased (after maximal biomass accumulation).

Further work by Hobbs *et al.* (1990) showed that the production of both actinorhodin and undecylprodigiosin was affected by only the nitrogen and phosphate sources. In a defined medium containing glucose and nitrate ions, undecylprodigiosin production was growth-associated while actinorhodin production was delayed until the growth rate decreased. Proline also permitted actinorhodin formation while ammonium salts did not. Actinorhodin production was prevented by as little as 1mM ammonium chloride while it required over 50mM ammonium chloride to inhibit undecylprodigiosin synthesis. The authors concluded that growth rate was not a controlling factor since glycine did not support actinorhodin formation although the growth rate on glycine varied by only 10% from that on proline. Phosphate had a less extreme effect on production. Actinorhodin synthesis was inhibited by 24mM phosphate while undecylprodigiosin production still occurred at this concentration. Since phosphate
affected antibiotic production even when grown in media containing nitrate ions as the nitrogen source, it was concluded that phosphate had a greater effect than nitrogen, but that regulation by these sources was interactive.

Subsequently, Hobbs et al. (1992) noted that secondary metabolite synthesis by *S. coelicolor* could be manipulated by 'physiological steering'. In 1990 these authors showed that undecylprodigiosin and actinorhodin production could be separated by increasing ammonium levels in the medium. In 1992, the authors demonstrated that exclusive methylenomycin synthesis could be obtained by the use of defined media containing glucose, alanine and elevated phosphate concentrations to prevent actinorhodin synthesis. Obanye et al. (1996) reported that methylenomycin production occurred when carbon flux through the pentose phosphate pathway increased relative to other carbon metabolic pathways. The authors noted the possibility that carbon metabolic pathways might be affected by the medium phosphate concentration.

More recently, Liao et al. (1995) analysed the relationship between actinorhodin production and growth on a large number of carbon and nitrogen sources. In defined media, sources supporting rapid growth (*e.g.* starch and glutamate) gave a biphasic production pattern; while sources supporting slower growth (*e.g.* maltose and nitrate) allowed production during rapid growth. The authors concluded that actinorhodin production by *S. coelicolor* was associated with sub-optimal growth rate.

These reports demonstrate that although high levels of carbon, nitrogen and phosphate permitting rapid growth may interfere with product biosynthesis, it is also possible to use these effects to manipulate *Streptomyces* metabolism and encourage the synthesis of a desired product rather than an unwanted one.
1.4 Further Fermentation Considerations: pH and Morphology

To achieve the best possible biomass and product yield from *Streptomyces*, growth conditions in liquid cultures (shake flasks or fermenters) must be optimised. This section will consider the effect of pH and hyphal morphology on growth and product formation by *Streptomyces* strains. These factors are closely related to those already considered in Sections 1.2 and 1.3 (acidification and carbon, nitrogen and phosphate sources). Other important parameters (*e.g.* temperature and oxygen) are not important here and will be mentioned only briefly when relevant.

1.4.1 Culture pH

The optimum pH for the growth of many micro-organisms, including streptomycetes, is pH 7.0. *Streptomyces* strains tend to have a broad optimal pH range and can grow between pH 5.0 and 9.0 (Bader, 1986). Extremes of either pH (acidic or basic) can degrade biological materials. Less extreme pH fluctuations can affect cell metabolism and integrity; affect the dissociation of organic acids; and reduce the activity of extracellular enzymes and the stability of secondary metabolites. Fluctuations in pH can also affect the rate of production. Examples are reviewed by Bader (1986).

Doull & Vining (1990a) note that a fall in pH can shift conditions away from the optima for production. Changes in pH may cause a reduction in growth rate and initiate secondary metabolism. Active acid production not only reduces the pH but affects the supply of precursors to desired products.

Chatterjee *et al.* (1983) observed that pH decreases in cultures of *S. venezuelae* (due to the production of pyruvic and α-ketoglutaric acids; Ahmed *et al.*, 1984) prevented both growth and chloramphenicol production. Further work with this strain was conducted with pH control (Shapiro & Vining, 1983, 1984).

Similarly, Wrigley-Jones *et al.* (1993) found that organic acid secretion by *S. lividans* TK24 reduced the pH to growth-inhibiting levels. Further cultures also required pH control. The authors suggested that the extracellular pH fluctuations (both the initial fall and the characteristic later rise in pH above 7.0) might affect the activity of secreted recombinant enzymes and products. Payne *et al.* (1990) and Erpicum *et al.* (1990) found that pH fluctuations affected the production, activity and stability of heterologous enzymes secreted by strains of *S. lividans* 66. Payne *et al.* (1990) showed that feeding carbon and nitrogen sources improved enzyme production more than simply controlling
the pH in a fermenter. This was probably due to a reduction in the conversion of 
substrates to acid by-products and more efficient synthesis of the desired product.

Bader (1986) notes that growth and production may occur at different pH values, 
but that it is more practical to compromise and regulate pH at a constant value. 
*Streptomyces* fermentations usually start at pH 6.5 to 7.0. The pH typically falls in the 
initial phase (growth) of the culture, followed by a pH increase (as seen by Wrigley-
Jones *et al.*, 1993).

It is not possible to actively or accurately control the pH of shake flasks, and 
buffering of pH fluctuations is dependent on the medium formulation. This may 
interfere with the organism. Phosphate buffers are popular (*e.g.* Chatterjee & Vining, 
1982), but may affect secondary metabolite formation, hence organic buffers such as 
MOPS can be used instead (*e.g.* Aharonowitz & Demain, 1977). Bader (1986) also 
notes that ammonium nitrate can be used both as a nitrogen source and to balance pH 
fluctuations (ammonium consumption decreases the pH, while nitrate uptake increases 
the pH). The same pattern of pH decrease and increase is true of combined sugars and 
amino acids in media. However, these sources may also affect other pathways (Section 
1.3) and the beneficial effects may be overshadowed by active acid secretion (*e.g.* 
Ahmed *et al.*, 1984; Section 1.2). In fermenters the pH can be monitored and controlled 
on-line via a pH electrode and the addition of acid or alkali to maintain a setpoint (*e.g.* 

### 1.4.2 Hyphal Morphology

Liquid cultures do not follow the same morphological cycle as cultures grown on 
solid surfaces (soil or agar). Ideally, they go through a series of comparable 
physiological changes starting with vegetative growth and switching to secondary 
metabolite synthesis when growth slows (Hopwood *et al.*, 1992). Secondary metabolite 
production may vary between solid and liquid cultures. Such differences are partly due 
to variations in hyphal morphology (Bushell, 1988). In liquid cultures *Streptomyces* 
morphology varies from mycelial fragments of 2 to 3\(\mu\)m in length, through dispersed 
mycelia, to hyphal pellets up to 5mm in diameter (Bushell, 1988; Whitaker, 1992).

In a culture of dispersed mycelia (ideally a homogeneous suspension of hyphae) 
there should be few limitations on substrate availability across the culture. However, 
mycelial cultures tend to be more viscous than unicellular cultures and increasing
viscosity reduces the mass transport of nutrients and oxygen (Prosser & Tough, 1991). In batch culture, the initial lag period is followed by a period of mycelial growth which may be exponential (e.g. the growth of *S. coelicolor* reported by Hobbs *et al.*, 1989) but may be quasi- or non-exponential (e.g. *S. cattleya* observed by Bushell & Fryday, 1983). Rapid growth is followed by a transition phase in which the growth rate slows due to nutrient and oxygen limitation, pH changes and other unfavourable factors. This is followed by stationary phase when cell growth and lysis are balanced but mycelia are metabolically active and engage in secondary metabolism and the synthesis of storage compounds. Finally, the culture enters the death stage when lysis exceeds growth.

Many *Streptomyces* strains grow as pelleted cultures. Pellets are more common in shake flasks than fermenters because of lower agitation and aeration which may encourage dispersed growth (Whitaker, 1992). The internal regions of pellets may become limited in the availability of nutrients and oxygen (Bader, 1986; Bushell, 1988; Prosser & Tough, 1991). Doull & Vining (1990a) point out that not only do pellets result in a heterogeneous environment for the constituent mycelia, but that interior mycelia may begin premature secondary metabolism due to nutrient limitation. Meanwhile the peripheral region of the pellet may still be engaged in rapid exponential growth (Bushell, 1988). Prosser & Tough (1991) observed that limitations can result in both reduced growth rate and lysis of the core biomass. Bader (1986) noted that *Streptomyces* require oxygen for growth and secondary product synthesis, but Bushell (1988) suggested that the interior cells will be oxygen-limited. This author also suggested that oxygen deficiency in pellets may be responsible for the production of TCA cycle-associated organic acids by cultures of filamentous organisms. Magnolo *et al.* (1991) thought that the expression of a bacterial haemoglobin by recombinant *S. lividans* may have improved oxygen uptake by internal, oxygen-deprived mycelia in pellets. It should be noted that streptomycetes form more loosely structured pellets than other filamentous organisms such as fungi (Bader, 1986) and the transfer of nutrients and oxygen occurs more freely in less dense pellets (Prosser & Tough, 1991).

Pellets are thought to be formed by either the entangling of hyphae into wefts then pellets, or by the clumping of spores and subsequent tangling of hyphae (Whitaker, 1992). Formation depends on a number of external factors. These include (Prosser & Tough, 1991; Whitaker, 1992) pH (in general pellets are formed at sub-optimal pH values and are less common at the optimum pH when dispersed growth is favoured);
medium composition (complex media usually support dispersed growth, while defined media support pelleted growth); inoculum size [most often a high inoculum concentration results in dispersed growth and a low concentration in pelleted growth (e.g. S. coelicolor; Hobbs et al., 1990)]; and other parameters including aeration, agitation and shear and viscosity. These are not generalisations and there are variations between strains. S. lividans is considered to form pellets in shake flasks (Hobbs et al., 1989; Whitaker, 1992). Magnolo et al. (1991) reported that S. lividans TK64 grew in clumps (pellets) even when flasks containing springs to promote dispersed growth.

In some cases pelleting may be desirable (for example, to promote organic acid production by fungi; Bushell, 1988). In the majority of situations, dispersed growth is required to minimise nutrient and oxygen deprivation (Whitaker, 1992). In 1982, Hodgson reported that dispersed growth of S. coelicolor A3(2) could be achieved by a combination of a stainless steel spring in each shake flask and the addition of PEG 6000 to minimal medium. An antifoaming agent was also needed. Doull & Vining (1989) obtained dispersed growth of S. coelicolor by the use of a defined medium containing 5% starch and 30mM glutamate as carbon and nitrogen sources. The authors reasoned that starch increased the medium viscosity and reduced the tendency of the organism to pellet in the same manner as PEG. Dispersed growth was aided by the addition of glass beads to shake flasks. These had a greater effect than springs and did not cause foaming. Hobbs et al. (1989) found that charged polymers such as Junlon were more effective than uncharged types like PEG in improving both biomass and product yield from S. lividans and S. coelicolor. It was thought that the polymers prevented the aggregation of spores and mycelia. However, it was not possible to remove the chemicals completely from the sampled biomass and variations occurred between batches.

In conclusion, a number of interconnected factors affect the biomass and product yield by Streptomyces. Changes in pH can occur due to acid secretion and can interfere with both growth and production. The pH may affect morphology which in turn affects growth and metabolism by the organism, and may itself cause acid production. S. lividans is particularly prone to pellet formation and this factor will be considered in Section 4 in light of the culture methods used during this project and the results presented in Section 3.
1.5 Methods for Organic Acid Identification

There are a number of methods available for the identification and measurement of organic acids. In some of the earliest reports on acid production by *Streptomyces* the authors (e.g. Hockenhull *et al.*, 1954; Doskocil *et al.*, 1959) describe the use of paper chromatography, following the derivatisation of excreted acids with aromatic compounds. Organic acids were identified by comparison with authentic standards; by which the amounts produced could also be calculated.

More recently, the use of alternative or more advanced chromatographic methods has been preferred. Ahmed *et al.* (1984) used a combination of column chromatography, thin-layer chromatography, nuclear magnetic resonance spectroscopy, enzymic assays and chemical assays to identify and measure the production of pyruvate, α-ketoglutarate and other organic acids by cultures of *S. venezuelae*. Payne *et al.* (1990) and DelaCruz *et al.* (1992) used gas chromatography (GC) to demonstrate that organic acids produced by a strain of *S. lividans* were isovalerate and acetate, rather than pyruvate and/or α-ketoglutarate. GC has also proved popular for the determination of acetate production by *E. coli* cultures (e.g. Brown *et al.*, 1977; Han *et al.*, 1992 and 1993).

By far the most commonly used method for analysing organic acids in the reports discussed in Section 1.2 has been high-performance liquid chromatography (HPLC). HPLC has certain advantages over other methods. This method is non-destructive and the separated components of a sample can be collected (as fractions) and used for further testing (Fallon *et al.*, 1987). Also, it is less time-consuming than methods requiring a number of preparative steps for analysis of a single compound, such as chemical and enzymic assays (Weigang *et al.*, 1989). Using HPLC it is possible to separate and analyse several disparate compounds simultaneously in a single run, usually lasting no more than 30 minutes depending on column and flow rate (Turner *et al.*, 1993; Drucker, 1987; Adams *et al.*, 1984). Sample preparation is simple and can require no more than filtration (e.g. El-Mansi & Holms, 1989; Hobbs *et al.*, 1992) or simple extraction (e.g. Surowitz & Pfister, 1985).

In 1987, Drucker noted that microbial acid products may be analysed by the use of either reversed-phase or cation-exchange HPLC columns. This author recommended the latter and particularly the Aminex HPX-87H organic acid analysis column. Without
exception, all of the workers cited in Section 1.2 using HPLC to analyse excreted acids used this column. HPX-87H has the advantage of being able to separate over 200 small organic compounds including organic acids and carbohydrates; and of being sensitive to nanogram levels of substances (Bio-Rad bulletin numbers 1847, 1833 and 1928; all available in 1996). When an Aminex HPX-87H column was combined with a suitable UV detector, Rumsby et al. (1987) were able to detect levels of just 25nmol lactate and 2.5nmol pyruvate by HPLC.

Surowitz & Pfister (1985) used an HPLC with an Aminex HPX-87H column to characterise organic acid production by *S. alboniger*. The identity of the excreted acid (pyruvate) was confirmed by the use of paper chromatography. Both Dekleva & Strohl (1987) and Hobbs et al. (1992) used an HPLC/Aminex HPX-87H system as the sole method for identifying and measuring the production of organic acids (including pyruvate and α-ketoglutarate) by *S. peucetius* and *S. coelicolor*. El-Mansi & Holms (1989) and Turner et al. (1993) used a combination of HPLC (with Aminex HPX-87H column) and enzymic assays to verify the identification and measurement of both excreted organic acid (acetate) and carbon sources (pyruvate and glucose) in samples from *E. coli* cultures.

It would appear to be wise to use a second method to confirm HPLC results obtained. Both Adams et al. (1984) and Rumsby et al. (1987) warn that use of an HPLC/Aminex HPX-87H system alone is not guaranteed 100% accuracy for identifying organic acids, especially when using complex mixtures. For this project HPLC was chosen as the preferred method for the identification and quantitation of organic acids excreted by *S. lividans* TK24. Two columns were used, one cation-exchange (Aminex HPX-87H) and one reversed-phase (Ultrasphere C18). Both were capable of separating organic acids and each provided a means of monitoring the utilisation of carbon sources from the medium (carbohydrates and amino acids respectively). Details of the columns and their use are given in Sections 2 and 3. Enzymic assays were used as a secondary method to confirm the identities of the organic acids produced by *S. lividans* TK24.
2 MATERIALS AND METHODS

2.1 Methods

The details of all major suppliers are given in Section 2.2.

2.1.1 Organism

*Streptomyces lividans* 66, strain TK24 which has the phenotype SLP2<sup>-</sup> SLP3<sup>-</sup> str-6 (Kieser et al., 1982; Hopwood et al., 1983b) was used throughout this project.

2.1.2 Spore Preparation

Sporulating cultures were grown from a spore suspension spread onto half-strength tryptone soya broth agar using a sterile platinum loop. This recipe was developed by French (1993) and was found to give a solid medium on which *S. lividans* grew and sporulated rapidly and reproducibly.

\[
\begin{align*}
\text{Tryptone soya broth (Oxoid)} & : 15\text{g L}^{-1} \\
\text{Bacteriological agar (Oxoid)} & : 20\text{g L}^{-1} \\
\text{Starch (Sigma)} & : 10\text{g L}^{-1}
\end{align*}
\]

The medium was made up to 1L with distilled water and autoclaved for 20 minutes at 121°C and 15psi pressure. To prevent fungal contamination cycloheximide (Sigma) was added before plates were poured at a concentration of 0.05mg·mL<sup>-1</sup>.

Agar cultures were incubated at 28°C for 7 days in an incubator (WTB Binder Labortechnik GmbH, Tuttlingen, Germany). Spores were harvested by the addition of 5mL 20% w/v glycerol (Fisons AnalaR) per plate followed by gentle scraping with a sterile disposable pipette to release the spores. Spore suspensions were pooled and aliquotted aseptically into 2mL portions in sterile Falcon tubes to provide consistent inocula throughout the experiments. Spore suspensions were stored at -20°C. The spore concentration was estimated and found to be approximately 2×10<sup>8</sup> spores per 2mL aliquot.
2.1.3 Shake Flask Cultures

Liquid cultures were grown in either defined or complex media or combinations of the two (details are given in Section 3).

A defined medium was developed based on previous recipes (Hopwood et al., 1985a) and which was called *Streptomyces* Minimal Medium (SMM). The basis of this medium was:

- NaH$_2$PO$_4$ (Sigma) 3g·L$^{-1}$
- K$_2$HPO$_4$ (BDH AnalR) 1.5g·L$^{-1}$
- MgSO$_4$·7H$_2$O (Fisons AnalR) 0.6g·L$^{-1}$
- FeSO$_4$·7H$_2$O (Fisons AnalR) 0.01g·L$^{-1}$

Carbon and nitrogen sources were added as described in Section 3, chosen from:

- (NH$_4$)$_2$SO$_4$ (Fisons AnalR)
- NaNO$_3$ (BDH AnalR)
- L-Alanine (Sigma)
- Glycine (Sigma)
- L-Glutamate, sodium salt (Sigma)
- L-Aspartate, sodium salt (Sigma)
- L-Lysine, hydrochloride (Sigma)
- D-Glucose (Fisons AnalR)
- Glycerol (Fisons AnalR)
- Maltose (BDH GPR)
- D-Fructose (BDH AnalR)
- Starch (BDH AnalR)

Glucose, maltose and fructose were autoclaved separately as 50% w/v solutions and added before inoculation as required. All others were added to the medium before sterilisation. The medium was made up to 1L with distilled water and autoclaved. Before inoculation the following solutions were added (all made up in distilled water and autoclaved separately):
(a) 10mL·L⁻¹ 10% w/v CaCl₂ (Fisons AnalaR)

(b) 2mL·L⁻¹ trace elements solution comprising:

- ZnCl₂ (Sigma) 40mg·L⁻¹
- FeCl₃·6H₂O (BDH AnalaR) 200mg·L⁻¹
- CuCl₂·2H₂O (BDH AnalaR) 10mg·L⁻¹
- MnCl₂·4H₂O (BDH AnalaR) 10mg·L⁻¹
- Na₂B₄O₇·10H₂O (BDH AnalaR) 10mg·L⁻¹
- (NH₄)₆Mo₇O₄·4H₂O (BDH AnalaR) 10mg·L⁻¹

(c) 1mL·L⁻¹ vitamins solution comprising:
- riboflavine (Sigma) 1g·L⁻¹
- niacinamide (Sigma) 1g·L⁻¹
- p-aminobenzoic acid (Sigma) 100mg·L⁻¹
- pyridoxine hydrochloride (Sigma) 500mg·L⁻¹
- thiamine hydrochloride (Sigma) 500mg·L⁻¹
- biotin (BDH) 200mg·L⁻¹

In all cases the pH of the medium was tested prior to autoclaving and was found to be pH 6.5 and was not adjusted further.

The complex medium used was called malt extract-peptone medium (MEP) and was modified from Reading & Cole (1977). This medium consisted of:

- Malt extract broth (Oxoid) 10g·L⁻¹
- Bacteriological peptone (Oxoid) 10g·L⁻¹
- Glycerol (Fisons AnalaR) 20g·L⁻¹

Where desired a phosphate supplement (MEP+10mM PO₄³⁻) was added as K₂HPO₄ (BDH AnalaR) at 1.74g·L⁻¹. The medium was made up to 1L with distilled water, adjusted to pH 7.0 when necessary and autoclaved. As described in Section 3, MEB and peptone were tested individually and were added to SMM (with additional nitrogen...
sources as listed in the text) at the above concentrations. Malt extract (Oxoid) was also used and was added at 10g·L⁻¹.

Cultures were grown in 2L Erlenmeyer flasks containing 200mL medium or 500mL medium for radiolabelling experiments. All flasks contained a stainless steel spring (Alliance Spring Co., London, UK) to reduce pelleting and were plugged with a foam bung to allow aeration. Flasks were inoculated aseptically with 5mL (into 500mL medium) or 2mL (into 200mL medium) thawed spore suspension, giving a final concentration of approximately 1×10⁶ spores per mL. Percentage germination of spores was not measured. Flasks were incubated in an orbital shaker (model ISF-IV, Kühner AG, Switzerland) set at 28°C and 200rpm for 7 days. Antifoam was not required. Each shake flask culture was tested for contamination at the end by plating a portion on ½TSB plates (Section 2.1.2). All of the cultures presented in Section 3 were free of contamination.

2.1.4 Dry Cell Weight Determination
Culture samples of 4mL were suction filtered through predried and preweighed AP25 prefilters (Millipore). The mycelium collected on each filter was washed with an equal volume of distilled water (this was tested and found to be sufficient to wash medium from biomass). The mycelium was dried at 100°C in an oven (WTB Binder Labortechnik GmbH, Tuttlingen, Germany) until constant weight. The filters were reweighed and the DCW determined as g·L⁻¹.

The filtrate collected was used for HPLC analysis, assuming that the concentration of medium components was diluted by half by the washing step (this was also tested and found to be an accurate assumption). Before filtering, the pH of samples was measured using pH sticks (BDH). Duplicate samples were taken at each time point and data presented in Section 3 is an average of these. Duplicates varied by no more than 0.5% at higher concentrations and 1.8% at lower values.

The statistical accuracy of this method was investigated and is reported in Appendix 1.
2.1.5 Deoxyribose Concentration Determination

The DNA content of mycelia was determined by measuring the deoxyribose concentration, to give an accurate indication of the switch between the growth and stationary phases in *S. lividans* cultures. Deoxyribose was assayed by a modified method based on the Burton (1956) method as reported by Hanson & Phillips (1981). This is the most commonly used method for estimation of DNA levels in cell extracts since it involves simple extraction, hydrolysis and colorimetric assaying of the pentose component of DNA. Methods for assaying complete DNA tend to be more complex. Since every base unit of DNA contains a deoxyribose residue, the quantitation of deoxyribose is a direct measure of DNA. Indeed, Burton (1956) refers to this method for the determination of DNA via deoxyribose quantitation.

1mL culture samples were harvested by centrifugation for 15 minutes at 8000xg in a Microfuge 11 (Beckman Instruments). The supernatant was discarded and the mycelium washed by resuspension in 0.1mL saline citrate solution [SSC: 8.75g sodium chloride (BDH AnalaR) and 4.41g trisodium citrate (Fisons AnalaR) made up to 1L with distilled water]. The sample was centrifuged for 15 minutes at 8000xg, the supernatant discarded and the pellets stored at -20°C until use.

Thawed samples were resuspended in 0.1mL SSC and acidified with 0.01mL 2.5M perchloric acid (Fisons AnalaR, 60% v/v). After cooling on ice for 30 minutes, samples were centrifuged for 10 minutes at 12000xg. The supernatants were discarded and the pellets resuspended in 0.1mL 0.5M perchloric acid. A further 0.7mL 0.5M perchloric acid was added to each sample, followed by heating in a waterbath at 70°C for 15 minutes, mixing every 5 minutes. Samples were centrifuged for 10 minutes at 5000xg and the supernatants transferred to Falcon tubes. The pellets were resuspended in 0.1mL SSC and extracted again. The total volume of the combined extracts from each sample were recorded.

Dilutions in a ten-fold range were prepared in 0.5M perchloric acid giving a final sample volume of 1mL. To each was added 2mL diphenylamine reagent [100mL acetic acid (BDH AnalaR), 1.5mL sulphuric acid (BDH AnalaR), 1.5g diphenylamine (BDH AnalaR) and on the day of use, 1.6mg·mL⁻¹ acetaldehyde (BDH GPR) per 20mL reagent]. Samples were incubated overnight at 28°C. The absorbance was read at 600nm
using a DU-64 spectrophotometer (Beckman Instruments) against a blank prepared with 0.5M perchloric acid. Standard curves were constructed (Figure 2.1) using pure deoxyribose (Sigma).

The statistical accuracy of this method was investigated and is reported in Appendix 2.

2.1.6 HPLC Analysis

High-performance liquid chromatography was used to identify medium components and measure their concentration by comparison with authentic standards.

All HPLC assays were performed using a System Gold Chromatographic system (Beckman Instruments). The system comprised a pump (model 126), autosampler (model 407), ultraviolet detector (model 166) and refractive index detector (model 156) connected via an analogue interface (model 406). Control and data logging facilities were provided by System Gold software run on an IBM computer.

All samples were prepared by filtering through a disposable 0.2μm pore PTFE syringe filter (Whatman). All eluents were filtered through a grade 5 qualitative cellulose filter paper, 2.5μm pore (Whatman) and degassed with helium (BOC, Guildford, Surrey, UK).

An Aminex HPX-37H cation exchange column was used to separate organic acids and carbohydrates. This column measured 300×7.8mm and was protected by a Cation H+ guard column (both from Bio-Rad). Samples of 0.02mL were loaded onto the column and eluted with 5mM sulphuric acid (BDH AnalRe; diluted with HPLC-grade water; approximately pH 2.0) at a flow rate of 0.6mL-min⁻¹. The column was maintained at a temperature of 50°C in a waterbath. Organic acids were detected by UV absorbance at 210nm and carbohydrates were detected by the RI detector at ×8.

An UltraspHERE ODS C18 analytical reversed phase column was used to separate simple aliphatic amino acids. This column measured 250×4.6mm and was protected by a 45×4.6mm column containing the same packing material (both from Beckman Instruments). Samples of 0.01mL were loaded onto the column and eluted with a 95:5% mixture of HPLC-grade water and HPLC-grade methanol (BDH HiPerSolv; approximately pH 2.5) at a flow rate of 0.5mL-min⁻¹. Both eluents contained 0.05%
orthophosphoric acid (BDH HiPerSolv) to prevent compounds sticking to the silica-based packing material. The column was maintained at a temperature of 25°C in a waterbath. Amino acids were detected by UV absorbance also at 210nm.

The columns were used independently and a standard column-switching protocol was followed throughout to prevent contamination or damage to the columns. The columns were maintained, cleaned and re-equilibrated as necessary and according to the manufacturer's instructions.

A series of organic acid standards were analysed to obtain retention times and standard curves of peak areas (concentrations between 0.001 and 1mg·mL⁻¹). These were used to identify and measure acids produced by \textit{S. lividans}. Sample standard curves are shown in Figures 2.3 and 2.4. The standards were:

- acetic acid (BDH)
- citric acid (BDH)
- formic acid (Sigma)
- fumaric acid (Aldrich)
- glyceric acid (Aldrich)
- glyoxylic acid (Sigma)
- isocitric acid (Sigma)
- α-ketoglutaric acid (Aldrich)
- lactic acid (Sigma)
- malic acid (Aldrich)
- oxaloacetic acid (Sigma)
- phosphoenolpyruvic acid (Sigma)
- pyruvic acid (Aldrich)
- succinic acid (Aldrich)

Standards were obtained as free acids or alkaline salts where necessary (also noted by Adams \textit{et al.}, 1984). All standards were 98+% pure, except isocitrate which was 95 to 98% pure. Standard dilutions were prepared in HPLC-grade water (following Pecina \textit{et al.}, 1984) for consistency between the two columns, and stored at 4°C or as indicated by the supplier. Small amounts of standards were used to 'spike' selected fermentation samples to aid identification of acid by-products. Standard preparations of medium components (amino acids, carbohydrates) were also analysed in order to measure the consumption of medium components by \textit{S. lividans}. Sample standard chromatograms are shown in Figures 2.5 and 2.6. Where desired, the eluate from the HPLC was collected using a fraction collector (model Frac 100, Pharmacia LKB Biotechnology, St. Albans, Hertfordshire, UK). Fractions containing individual medium components or acids produced by the organism were used for further tests as described in Sections 2.1.9 and 2.1.11. The statistical accuracy of the HPLC analysis was investigated and is reported in Appendix 3.
Figure 2.1: Example of a standard curve used to determine deoxyribose concentration.

Figure 2.2: Example of a standard curve used to determine glucose concentration.
Figure 2.3: Example of a standard curve used to calculate pyruvate concentration.

Figure 2.4: Example of a standard curve used to calculate α-ketoglutarate concentration.
Figure 2.5: Example of a chromatogram of a separation of mixed standards at various concentrations using the Aminex HPX-87H column with UV (—) and RI (——) detectors. 1 a-ketoglutarate UV (7.90 minutes); 2 a-ketoglutarate RI (8.10 min); 3 glucose RI (8.95 min); 4 pyruvate UV (9.00 min); 5 pyruvate RI (9.20 min); 6 lactate UV (12.30 min); 7 lactate RI (12.60 min); 8 glycerol RI (13.20 min); 9 acetate UV (15.00 min); 10 acetate RI (15.10 min).

Figure 2.6: Example of a chromatogram of a separation of mixed standards at various concentrations using the Ultrasphere ODS CI8 column with UV detection only. 1 alanine (5.25 min); 2 glutamate (5.40 min); 3 pyruvate (7.80 min).
2.1.7 Glucose Analysis

To check the accuracy of measurement of glucose by HPLC analysis, a selection of samples from each culture containing glucose were assayed by a modified o-toluidine method using Sigma kit number 635 (Sigma).

1mL culture samples were centrifuged for 5 minutes at 10000xg. Portions of 0.12mL supernatant were added to 1.08mL trichloroacetic acid, mixed and allowed to stand for 5 minutes to precipitate proteins. The samples were centrifuged for a further 5 minutes at 10000xg to obtain a clear supernatant. 1mL supernatant from each sample was added to 5mL o-toluidine reagent in a test tube and boiled at 100°C for 10 minutes. The tubes were cooled in water for 3 minutes and the samples transferred into cuvettes. The absorbance of each was read at 635nm using a DU-64 spectrophotometer (Beckman Instruments) against a blank prepared with water/TCA and a 1mg-mL^-1 glucose standard prepared with each batch of samples. Standard curves were constructed (Figure 2.2) using pure glucose solutions supplied with the kit.

Comparison of glucose values obtained from HPLC and o-toluidine assays for selected samples showed that the measurements differed by an average of less than 4%. It was concluded that the HPLC-based measurement of carbohydrates was accurate.

2.1.8 Ammonium and Nitrate Analysis

Ammonium (NH₄⁺) ions were assayed in culture samples following the phenol-hypochlorite method of Weatherburn (1967). Culture samples of 1mL were centrifuged for 5 minutes at 10000xg. To portions of 0.02mL supernatant were added 5mL aliquots of phenol-nitroprusside reagent [10g phenol (BDH AnalR) and 5mg sodium nitrosopentacyanoferrate(III) (BDH AnalR) made up to 1L with HPLC-grade, ammonium-free water]. After mixing, 5mL alkaline hypochlorite reagent [5g sodium hydroxide (BDH AnalR) and 8.4mL sodium hypochlorite (BDH Spectrosol) made up to 1L with HPLC-grade water] was added to each sample. After further mixing samples were incubated for 15 minutes at 37°C. The absorbance was read at 625nm using a DU-64 spectrophotometer (Beckman Instruments) against a blank prepared with ammonium-free water.
No reliable assay method could be found for nitrate (NO$_3^-$) ions due to interference by other factors. However, this ion was found to have a high absorbance at 210nm in HPLC samples, therefore an analysis of nitrate presence in media was done qualitatively using HPLC. It was found that nitrate was poorly used by *S. lividans* and was present throughout all cultures to which it was added.

### 2.1.9 Enzymic Assays for Pyruvate and α-Ketoglutarate

To confirm the identity of the organic acids produced by cultures of *S. lividans*, fractions containing each acid were prepared by HPLC (Section 2.1.6) and assayed enzymically. The assay methods were tested on authentic standards before application to separated samples.

Selected samples from each acid-producing culture were assayed against control samples (prepared using HPLC eluent), standards and samples from non-acid-producing cultures. Pyruvate was assayed using Sigma kit number 726 (Sigma). This kit contains L-lactate dehydrogenase (EC 1.1.1.27). The assay depends on the UV spectrophotometric reaction at 340nm resulting from the conversion of NADH to NAD on addition of the enzyme to the substrate, as follows (NADH absorbs at 340nm while NAD does not):

\[
\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{L-lactate} + \text{NAD}^+ 
\]

(Equation 2.1)

The kit instructions were adapted to account for the small size (0.4mL) of fractions obtained containing the whole pyruvate peak but no other contaminants. Each sample was made up to 1.2mL with 8% w/v perchloric acid (Fisons AnalaR). From each sample, 1mL supernatant was placed into a cuvette prewarmed at 37°C in a DU-70 scanning spectrophotometer (Beckman Instruments). To this was added 0.25mL Trizma base buffer and 0.25mL of a 0.45mg-mL$^{-1}$ NADH solution in buffer from the kit. The sample was quickly mixed, warmed and the absorbance recorded. The pH of this mixture was approximately 7.5 in all cases. 0.01mL LDH was added and the decrease in absorbance at 340nm scanned. Examples of positive and negative responses are shown in Figure 2.7.
\( \alpha \)-Ketoglutarate was assayed by a method based on Bergmeyer (1974). This assay depends on the same reaction at 340nm resulting from the conversion of NADH to NAD following the addition of glutamate dehydrogenase (EC 1.4.1.3) to the substrate in the presence of ammonium ions, as follows:

\[
\alpha\text{-ketoglutarate} + \text{NH}_4^+ + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{glutamate dehydrogenase}} \text{L-glutamate} + \text{NAD}^+ + \text{H}_2\text{O}
\]

(Equation 2.2)

The method was adapted to account for the low concentration of \( \alpha \)-ketoglutarate produced by the organism and the acidity of the sample. Into a cuvette prewarmed to 25°C in the DU-70 scanning spectrophotometer was placed 2.5mL 0.1M (pH 7.3) Imidazole buffer (Sigma), 0.1mL 10mg·mL\(^{-1}\) EDTA solution (Fisons AnalR), 0.03mL 0.1M ADP solution (Sigma), and 0.03mL 5mg·mL\(^{-1}\) NADH solution in buffer. After mixing, 0.05mL 12.8M ammonium acetate (Fisons AnalR) was added. After further mixing, 0.4mL sample was added. The absorbance of the sample was recorded. The pH of this mixture was approximately 7.3 in all cases. Finally 0.01mL GDH was added and the decrease in absorbance at 340nm scanned. Examples of positive and negative responses for this assay are shown in Figure 2.8.

### 2.1.10 Carbon and Nitrogen Analysis

Total carbon was measured in culture samples using a Total Organic Carbon analyser (model TOC-5050, Shimadzu Corporation, Kyoto, Japan). This equipment analyses liquid samples, which are diluted with distilled water such that the carbon content is within the range of preset calibration curves. Samples of 1mL were suitably diluted and used to measure the carbon in the whole broth (medium and biomass). Identical 1mL samples were centrifuged for 5 minutes at 10000×g and the supernatant was transferred, diluted and used to measure carbon in the medium. The biomass pellet was resuspended in 1mL distilled water, diluted and used to measure carbon in the biomass. Samples were injected into the analyser according to the operating instructions and combusted in a furnace set at 680°C. Carbon was determined as parts per million (ppm) and converted to g·L\(^{-1}\). The analyser took up to 5 injections consecutively from each sample and rejected it if the readings were not within a standard deviation of 2%. In addition, the statistical accuracy of the whole method was investigated and is reported in Appendix 4.
Figure 2.7: Examples of positive and negative responses from pyruvate assays.  
*a*: sample containing pyruvate (54h of an SMM-glucose-aspartate-nitrate culture);  
*b*: sample from a non-acid-producing culture (54h of an SMM-glucose-aspartate-ammonium culture);  
*c*: control sample.
Figure 2.8: Examples of positive and negative responses from α-ketoglutarate assays.  
a: sample containing α-ketoglutarate (78h of an SMM-glucose-aspartate culture);  
b: control sample.
Total nitrogen was analysed using a Nitrogen Determinator (model FP-428, Leco Corporation, Michigan, USA). Samples of whole broth, medium and suspended biomass were prepared as described for carbon analysis, but were not diluted. Each 1mL sample (or 2mL sample for defined media) was placed in a predried and preweighed foil cup and dried overnight at 100°C in the oven described in Section 2.1.4. Samples were loaded into the machine and combusted at 850°C according to the operating instructions. Nitrogen was determined as a percentage of weight, which was converted into g·L⁻¹. As the samples were combusted, only a single reading was taken from each. The statistical accuracy of this method was investigated and is reported in Appendix 4.

2.1.11 Radiolabelled Materials

D-[U-¹⁴C]glucose (specific activity >250mCi·mmol⁻¹) was obtained from Amersham and was added to defined media (described in Section 3) at a concentration of 0.33µCi·mL⁻¹. L-[U-¹⁴C]alanine (specific activity >150mCi·mmol⁻¹) and L-[U-¹⁴C]aspartate (specific activity >200mCi·mmol⁻¹) were also obtained from Amersham and were added to defined media at a concentration of 0.25µCi·mL⁻¹.

To analyse radioactivity present in either medium components or organic acids produced by the organism, fractions of samples separated by HPLC were added to 2mL portions of Ecoscint A scintillation solution (National Diagnostics). Samples were mixed well and the radioactivity measured with a Liquid Scintillation Counter (model 1900CA, Packard Instruments, Pangbourne, Berkshire, UK) as counts per minute (cpm). After separation, portions of the HPLC eluate were examined and were found to be free of radioactive contamination.

At the end of each culture, the radioactivity present in the biomass and as dissolved CO₂ in the broth was measured. From this, the amount lost to CO₂ via respiration could be estimated. Biomass samples were washed, dried and resuspended in distilled water according to instructions accompanying NCS Tissue Solubiliser (Amersham). After transferring the samples to suitable counting vials, NCS was added in a ratio of 6 parts NCS to 1 part sample. The vials were heated in a waterbath set at 50°C until completely homogeneous. After cooling, 2mL Ecoscint A was added to each and the radioactivity measured as before.
Dissolved CO₂ was released from 50mL broth portions by acidification with 5mL 1M sulphuric acid (BDH AnalaR). Released CO₂ was captured overnight in 5mL Hyamine hydroxide (methyl benzethonium hydroxide; Sigma). Portions of Hyamine hydroxide were added to 2mL Ecoscint A in counting vials and the radioactivity measured as before.

Analysis of data showed the biomass and dissolved CO₂ methods to be accurate. Comparison of data also showed that identical fractions collected from repeat injections of samples varied by less than 6.2%; and that repeat counts by the Liquid Scintillation Counter varied by less than 2.5%.

2.1.12 Fermentations
Fermentations were conducted in a 2L capacity fermenter (LH Fermentation, Berkshire, UK). The vessel was filled with 1.35L of medium, assembled and autoclaved for 20 minutes at 121°C and 15psi pressure. After cooling, the vessel and probes were attached to the controls and allowed to equilibrate overnight. A sterile air supply was connected and the air flow rate set at 2.0L-min⁻¹ (1vvm). Exhaust air passed through a condenser to mass spectrometer equipment. The agitation rate was set at 1000rpm and the temperature at 28°C. The pH was set at 7.0 and controlled automatically from a reservoir of sterile 4M sodium hydroxide (BDH AnalaR) where desired. Manual additions from a reservoir of 0.01% PPG antifoam (BDH) were made to control foaming when necessary.

The fermenter was inoculated aseptically with a 10% v/v culture, combined at the time of addition with any further medium components required. The total volume was 1.5L. The inoculum was grown for 24h or 48h in the same medium as the fermenter, as described in Section 2.1.3. Data was logged using RT-DAS software run on an IBM computer. Exhaust gas analysis was logged on the same system.
### 2.2 Suppliers

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma</td>
<td>Poole, Dorset, UK.</td>
</tr>
<tr>
<td>BDH</td>
<td>Poole, Dorset, UK.</td>
</tr>
<tr>
<td>Fisons</td>
<td>Loughborough, Leicestershire, UK.</td>
</tr>
<tr>
<td>Aldrich</td>
<td>Gillingham, Kent, UK.</td>
</tr>
<tr>
<td>Oxoid</td>
<td>Basingstoke, Hampshire, UK.</td>
</tr>
<tr>
<td>Whatman</td>
<td>Maidstone, Kent, UK.</td>
</tr>
<tr>
<td>Millipore</td>
<td>Watford, Hertfordshire, UK.</td>
</tr>
<tr>
<td>Beckman Instruments</td>
<td>High Wycombe, Buckinghamshire, UK.</td>
</tr>
<tr>
<td>Bio-Rad</td>
<td>Hemel Hempstead, Hertfordshire, UK.</td>
</tr>
<tr>
<td>Boehringer Mannheim</td>
<td>Lewes, East Sussex, UK.</td>
</tr>
<tr>
<td>Amersham</td>
<td>Little Chalfont, Buckinghamshire, UK.</td>
</tr>
<tr>
<td>National Diagnostics</td>
<td>Atlanta, Georgia, USA.</td>
</tr>
</tbody>
</table>
3 RESULTS

3.1 Identification of Organic Acids (Results and Discussion)

Throughout this project the principal method used for the characterisation of organic acids produced by *Streptomyces lividans* TK24 was HPLC. The position (retention time) and size (peak area) of peaks on the resulting chromatograms can be used to identify and quantify compounds respectively, based on comparison with authenticated standards. The following sub-section concerns details of this method and serves as an introduction for subsequent sections (3.2-3.8).

Two HPLC columns were used (described in Section 2.1.6), an Aminex HPX-87H cation-exchange column and an Ultrasphere ODS C18 reversed-phase column. Both columns were capable of determining organic acids when combined with a UV detector set at a wavelength of 210nm. Statistical analysis of a number of standards showed that the identification and measurement of acids was more accurate using the Aminex column (see Tables 3.1 and 3.2 and Appendix 3). All organic acid data presented in Section 3 was calculated from chromatograms derived from runs using the Aminex column.

The Aminex column was also useful for reliably analysing carbohydrate medium components when combined with a suitable RI detector (see Table 3.3). Carbohydrates were identified and measured against standard solutions.

The Ultrasphere column was used as an alternative for two main reasons: (i) it provided a convenient confirmation of organic acid identities in addition to results from the Aminex column; and (ii) it was capable of accurately and reproducibly separating the simple aliphatic amino acids used as medium components (see Table 3.4). Amino acids were also identified and measured against standard solutions. These compounds absorb at 210nm, simplifying the analytical process.

After testing individual organic acid standards, separation of standard mixtures was optimised for each column. The column temperature, flow rate and eluent composition were adjusted to achieve the best possible separation.

The retention times obtained for the standards using each column are shown in Tables 3.1 and 3.2. The times obtained from the Aminex column were checked against
the available technical data (Bio-Rad bulletin 1847; Adams et al., 1984; Rumsby et al., 1987) and found to be accurate. A peculiar anomaly was encountered with oxaloacetate, which is sometimes called oxalacetate. The Bio-Rad data refers to both types, with technical data for the latter indicating a retention time of 7.36 minutes for the system used for this project. No technical data is given for the former type. Analysis of the standard obtained from Sigma revealed a retention time of 9.00 minutes, co-eluting with pyruvate at 9.05 minutes. Rumsby et al. (1987) also refer to two types of this acid, oxaloacetate I and oxaloacetate II. Comparison of published data with results obtained from this project indicated that oxaloacetate I is equivalent to the compound referred to by Bio-Rad as oxalacetate and that oxaloacetate II is equivalent to oxaloacetate. All forms of oxaloacetate obtained for this work appear to correspond to type II. Although the co-elution of oxaloacetate type II complicated analysis of pyruvate using the Aminex column, it was found that oxaloacetate and pyruvate eluted separately on the Ultrasphere column, demonstrating the advantage of using two columns for analysis. It can be speculated that at the pH used for the mobile phase (pH 2.0-2.5) a significant amount of oxaloacetate existed in a protonated form. According to Dawson et al. (1986) the pKa of oxaloacetate is 2.22 and this compound can exist in at least three forms (hydrate, keto and enol).

The retention times shown in Tables 3.1 and 3.2 were obtained from the analysis of individual standards. These were found to be consistent in mixtures of standards where co-elution was unavoidable. In all cases, including the pyruvate-oxaloacetate pair, identification of acids was confirmed by the independent use of the two columns. The use of retention times to identify acid products and medium components was found to be accurate and reproducible based on statistical analysis (Tables 3.1 to 3.4) and extensive tests of standard mixtures.

Acid concentrations were measured based on the peak areas of standards. Standard curves were constructed using System Gold software and manually from the peak areas generated by analysis of a range of concentrations of each standard (0.001 to 1mg-mL⁻¹). The use of peak areas to measure the concentrations of acid products and medium components was found to be accurate and reliable based on statistical analysis (Tables 3.1 to 3.4) and tests using various dilutions.
It is possible to use the ratios of peak areas from UV and RI analysis as a further parameter for the analysis of organic acid peaks, particularly peak purity (Weigang et al., 1987; Masson et al., 1991; Bio-Rad bulletin 1847). It has, however been noted that refractometry may not always be useful since, although all compounds have a refractive index, the difference in RI between the sample and the mobile phase may be too small to detect (Bidlingmeyer, 1992). Weigang et al. (1987) also found that the UV detector response was more accurate and reproducible, and up to 600 times more sensitive for many organic acids compared to an RI detector. The RI detector was unable to detect low levels of citrate and fumarate in fermentation samples, while results from the UV detector indicated that 0.02mM citrate and 0.008mM fumarate were present.

Analysis of the peak areas obtained for organic acid standards from UV and RI detectors connected in series showed that the UV detector used for this project was up to 1600 times more sensitive (and at least 20 times more sensitive) than the RI detector. Some examples are given in Table 3.5. Masson et al. (1991) note that UV detection is required for compounds with low RI responses, including pyruvate.

Since the levels of organic acids produced by S. lividans TK24 were low at most points throughout each fermentation (less than 10mM, details in Sections 3.2 to 3.8), UV was chosen as the principal detection method. Where possible, \( \frac{UV}{RI} \) ratios were checked against those obtained for the standards. Otherwise, peak purity was tested by the independent use of both columns, co-chromatography of samples with standards and by enzymic assays. The RI detector was important for the detection of carbohydrates, which do not have high UV absorbance (Weigang et al., 1989).

Fermentation samples were not pretreated except for filtering (Sections 2.1.4 and 2.1.6), as recommended by the column manufacturers. Adams et al. (1984) reported that the complexity of chromatograms from fermentation samples affected both the identification and quantification of medium components. During this project it was found that chromatograms of separations using the Aminex column were simple in comparison to those published in the above report for both SMM and MEP-based media. The chromatograms were suitable for the characterisation of acid production by S. lividans. Sample chromatograms are shown in Figures 3.1 and 3.2. Analysis of SMM-based media using the Ultrasphere column also gave simple chromatograms, although those from MEP-based media were more complex when peptone was present (see Section 3.5).
It was found that *S. lividans* TK24 produced exclusively pyruvate and α-ketoglutarate. No other acids were detected. It is worth noting that no isovalerate was detected (elution time of standard was 24.26 minutes with the Aminex column), in comparison to the findings of DelaCruz *et al.* (1992) who studied the parent strain *S. lividans* 66. Acid identities were subsequently confirmed by enzymic assays on fractions of separated samples (Section 2.1.9). Also, ‘spiking’ tests were carried out, where selected fermentation samples suspected to contain pyruvate and α-ketoglutarate were co-chromatographed with known amounts of pyruvate and α-ketoglutarate standards. In all cases, and with both columns, the suspected acid and the added standard formed a single peak; and the sum of the calculated concentration of the produced acid and the amount of added standard agreed with the amount in the ‘spiked’ peak. For example:

SMM-glucose-alanine medium at 54h

- Suspected pyruvate peak 0.2729 mg·mL⁻¹
  + pyruvate standard 0.0500 mg·mL⁻¹
  expected final concentration 0.3229 mg·mL⁻¹
  actual final concentration 0.3245 mg·mL⁻¹

- Suspected α-ketoglutarate peak 0.1574 mg·mL⁻¹
  + α-ketoglutarate standard 0.0700 mg·mL⁻¹
  expected final concentration 0.2274 mg·mL⁻¹
  actual final concentration 0.2223 mg·mL⁻¹

Other standards with similar retention times were also used to ‘spike’ samples. Oxaloacetate, malate, glyoxylate and isocitrate all formed double peaks or shoulders on the suspected pyruvate peak (depending on the column used since oxaloacetate co-eluted with pyruvate using the Aminex column). Acetate and isocitrate all formed double peaks or shoulders on the suspected α-ketoglutarate peak.

Based on these observations, it was concluded that a combination of HPLC and enzymic assays was suitable for the identification and measurement of organic carbon and nitrogen substrates and organic acid by-products during cultures of *S. lividans* TK24.
<table>
<thead>
<tr>
<th>Average Retention Time</th>
<th>Compound</th>
<th>Accuracy of Retention Time</th>
<th>Accuracy of Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.63</td>
<td>phosphoenolpyruvate</td>
<td>0.025%</td>
<td>0.42%</td>
</tr>
<tr>
<td>7.67</td>
<td>citrate</td>
<td>0.028%</td>
<td>0.19%</td>
</tr>
<tr>
<td>7.72</td>
<td>isocitrate</td>
<td>0.008%</td>
<td>0.24%</td>
</tr>
<tr>
<td>7.90</td>
<td>α-ketoglutarate</td>
<td>0.015%</td>
<td>0.40%</td>
</tr>
<tr>
<td>9.00</td>
<td>oxaloacetate II</td>
<td>0.015%</td>
<td>0.19%</td>
</tr>
<tr>
<td>9.05</td>
<td>pyruvate</td>
<td>0.009%</td>
<td>0.08%</td>
</tr>
<tr>
<td>9.16</td>
<td>glyoxylyte</td>
<td>0.015%</td>
<td>0.20%</td>
</tr>
<tr>
<td>9.25</td>
<td>malate</td>
<td>0.017%</td>
<td>0.43%</td>
</tr>
<tr>
<td>10.51</td>
<td>glycerate</td>
<td>0.022%</td>
<td>0.26%</td>
</tr>
<tr>
<td>11.52</td>
<td>succinate</td>
<td>0.012%</td>
<td>0.20%</td>
</tr>
<tr>
<td>12.25</td>
<td>lactate</td>
<td>0.025%</td>
<td>0.35%</td>
</tr>
<tr>
<td>13.56</td>
<td>formate</td>
<td>0.019%</td>
<td>0.29%</td>
</tr>
<tr>
<td>14.01</td>
<td>fumarate</td>
<td>0.018%</td>
<td>0.09%</td>
</tr>
<tr>
<td>14.84</td>
<td>acetate</td>
<td>0.004%</td>
<td>0.40%</td>
</tr>
</tbody>
</table>

Table 3.1: Analysis of organic acid standards using an Aminex HPX-87H column with a UV detector at A<sub>210</sub>. All standards 0.5mg-mL<sup>-1</sup>, except fumarate and phosphoenolpyruvate at 0.1mg-mL<sup>-1</sup>.

† Minutes from injection.
‡ Statistical analysis of 5 repeat injections with 95% confidence limits of the mean expressed as a percentage of the mean.

<table>
<thead>
<tr>
<th>Average Retention Time</th>
<th>Compound</th>
<th>Accuracy of Retention Time</th>
<th>Accuracy of Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.10</td>
<td>glyoxylyte</td>
<td>0.027%</td>
<td>0.25%</td>
</tr>
<tr>
<td>6.59</td>
<td>glycerate</td>
<td>0.120%</td>
<td>1.76%</td>
</tr>
<tr>
<td>6.92</td>
<td>formate</td>
<td>0.083%</td>
<td>0.22%</td>
</tr>
<tr>
<td>7.15</td>
<td>phosphoenolpyruvate</td>
<td>0.043%</td>
<td>0.59%</td>
</tr>
<tr>
<td>7.63</td>
<td>malate</td>
<td>0.021%</td>
<td>0.37%</td>
</tr>
<tr>
<td>7.82</td>
<td>pyruvate</td>
<td>0.079%</td>
<td>0.61%</td>
</tr>
<tr>
<td>7.85</td>
<td>isocitrate</td>
<td>0.024%</td>
<td>2.04%</td>
</tr>
<tr>
<td>7.90</td>
<td>oxaloacetate II</td>
<td>0.091%</td>
<td>6.04%</td>
</tr>
<tr>
<td>8.60</td>
<td>lactate</td>
<td>0.065%</td>
<td>0.35%</td>
</tr>
<tr>
<td>9.03</td>
<td>acetate</td>
<td>0.049%</td>
<td>0.72%</td>
</tr>
<tr>
<td>9.46</td>
<td>α-ketoglutarate</td>
<td>0.062%</td>
<td>0.45%</td>
</tr>
<tr>
<td>11.94</td>
<td>citrate</td>
<td>0.200%</td>
<td>0.76%</td>
</tr>
<tr>
<td>12.65</td>
<td>succinate</td>
<td>0.400%</td>
<td>1.19%</td>
</tr>
<tr>
<td>14.25</td>
<td>fumarate</td>
<td>0.130%</td>
<td>0.55%</td>
</tr>
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</table>

Table 3.2: Analysis of organic acid standards using an Ultrasphere C18 column with a UV detector at A<sub>210</sub>. All standards 0.5mg-mL<sup>-1</sup>, except fumarate at 0.1mg-mL<sup>-1</sup>.

† Minutes from injection.
‡ Statistical analysis of 5 repeat injections with 95% confidence limits of the mean expressed as a percentage of the mean.
Table 3.3: Analysis of carbohydrate standards using an Aminex HPX-87H column with an RI detector at x8. Glucose and maltose 15mM, fructose 30mM and glycerol 60mM. † Minutes from injection. ‡ Statistical analysis of 5 repeat injections with 95% confidence limits of the mean expressed as a percentage of the mean.

<table>
<thead>
<tr>
<th>AVERAGE RETENTION TIME †</th>
<th>COMPOUND</th>
<th>ACCURACY OF RETENTION TIME ‡</th>
<th>ACCURACY OF PEAK AREA ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.00</td>
<td>starch</td>
<td>0.010%</td>
<td>0.79%</td>
</tr>
<tr>
<td>7.32</td>
<td>maltose</td>
<td>0.027%</td>
<td>0.45%</td>
</tr>
<tr>
<td>8.95</td>
<td>glucose</td>
<td>0.019%</td>
<td>0.43%</td>
</tr>
<tr>
<td>9.57</td>
<td>fructose</td>
<td>0.009%</td>
<td>0.23%</td>
</tr>
<tr>
<td>13.20</td>
<td>glycerol</td>
<td>0.023%</td>
<td>1.19%</td>
</tr>
</tbody>
</table>

Table 3.4: Analysis of amino acid standards using an Ultrasphere C18 column with a UV detector at A_210. All standards 15mM. † Minutes from injection. ‡ Statistical analysis of 5 repeat injections with 95% confidence limits of the mean expressed as a percentage of the mean.

<table>
<thead>
<tr>
<th>AVERAGE RETENTION TIME †</th>
<th>COMPOUND</th>
<th>ACCURACY OF RETENTION TIME ‡</th>
<th>ACCURACY OF PEAK AREA ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.86</td>
<td>lysine</td>
<td>0.037%</td>
<td>0.27%</td>
</tr>
<tr>
<td>5.15</td>
<td>glycine</td>
<td>0.034%</td>
<td>0.34%</td>
</tr>
<tr>
<td>5.28</td>
<td>alanine</td>
<td>0.024%</td>
<td>0.31%</td>
</tr>
<tr>
<td>5.32</td>
<td>aspartate</td>
<td>0.029%</td>
<td>0.29%</td>
</tr>
<tr>
<td>5.42</td>
<td>glutamate</td>
<td>0.018%</td>
<td>0.48%</td>
</tr>
</tbody>
</table>

Table 3.5: Examples of $\frac{UV}{RI}$ ratios for organic acid standards. Data are averages of 5 injections. ND=not detected.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>UV RET. TIME</th>
<th>UV PEAK AREA</th>
<th>RI RET. TIME</th>
<th>RI PEAK AREA</th>
<th>$\frac{UV}{RI}$ RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyruvate</td>
<td>9.09</td>
<td>164.542</td>
<td>9.50</td>
<td>0.592</td>
<td>277.94</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>7.91</td>
<td>146.213</td>
<td>8.32</td>
<td>0.718</td>
<td>203.64</td>
</tr>
<tr>
<td>oxaloacetate</td>
<td>9.03</td>
<td>59.120</td>
<td>9.44</td>
<td>0.036</td>
<td>1642.22</td>
</tr>
<tr>
<td>citrate</td>
<td>7.69</td>
<td>19.590</td>
<td>8.10</td>
<td>0.621</td>
<td>31.55</td>
</tr>
<tr>
<td>acetate</td>
<td>14.87</td>
<td>7.797</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 3.1: Examples of analyses using the Aminex HPX-87H column: (a) sample taken at 78h of a culture grown in SMM containing 0.5% w/v starch and 15 mM aspartate; (b) sample taken at 72h of a culture grown in SMM containing 10 g L⁻¹ malt extract and 15 mM aspartate. Traces were similar for media containing malt extract broth or full MEP medium. It can be seen that sugars (RI ———) and organic acids (UV ———) were clearly separated.
Figure 3.2: Examples of analyses using the Ultrasphere ODS C18 column: (a) sample taken at 0h; and (b) sample taken at 48h of a culture grown in SMM containing 30mM glucose and 15mM aspartate. It can be seen that amino acids and organic acids were clearly separated.
3.2 Shake Flask Cultures Using *Streptomyces* Minimal Medium Containing Glucose and Defined Nitrogen Sources

3.2.1 Objective

The aim of this set of shake flasks was to characterise acid production (identities and quantities) when *S. lividans* TK24 was grown in defined medium containing simple carbon and nitrogen sources. Glucose was chosen as the primary carbon source since this is a commonly-used source in microbial fermentations (see Section 3.4 for tests using alternative carbon sources in light of the carbon effect described in Section 1.3). A variety of nitrogen sources were tested to investigate their influence on metabolism and acid production, in respect of the nitrogen effect discussed in Section 1.3.

Where added, the initial glucose concentration was consistent throughout this set of shake flasks (5g L\(^{-1}\)), which supplied 2.0g L\(^{-1}\) of carbon at the start of each culture. In cultures containing glucose, the amount of each nitrogen source added was also kept constant for simplicity. A summary of the media used in this set of shake flasks is given in Table 3.6.

In media containing glucose and an inorganic nitrogen source (media 1 and 2; initial nitrogen 0.42g L\(^{-1}\)) it was found that nitrogen was in excess throughout the duration of each culture. Further media tested contained glucose and amino acids (media 3-7), which acted as primary nitrogen and secondary carbon sources. Here, it was thought reasonable to reduce the initial nitrogen concentration by half to 0.21g L\(^{-1}\). This ensured that the ‘standard’ initial carbon level of 2.0g L\(^{-1}\) was not greatly exceeded when glucose was kept at a constant concentration. The exception to this rule was glucose-lysine medium (7). When lysine was added at 7.5mM (giving total initial carbon of 2.54g L\(^{-1}\) and initial nitrogen of 0.21g L\(^{-1}\), in keeping with other glucose-amino acid media), the culture failed to grow. Further tests with a doubled amount of lysine (15mM) gave cultures which exhibited poor growth but significant acid production. The results of this combination are presented here. In contrast to media containing glucose and an inorganic nitrogen source, most of the organic nitrogen sources were utilised completely. It is possible that these cultures were nitrogen-limited and this is considered later in this section.

For media containing mixed nitrogen sources (media 8-11), it was decided to maintain the initial levels of carbon sources (glucose and amino acids) previously used
and to study the effect of extra nitrogen on acidification. However, in media without glucose (media 12-19) it was necessary to increase the amounts of amino acids added to maintain the initial carbon at a suitable level (approximately 2.0g·L\(^{-1}\)). In these cultures the initial nitrogen varied considerably.

A similar approach to medium formulation was adopted by Ahmed et al. (1984) and Hobbs et al. (1992). Ahmed et al. (1984) kept the initial glucose consistent and varied the organic and inorganic nitrogen sources to supply 30 or 60mM initial nitrogen. No mention was made by these authors of the effect of the extra carbon supplied by the organic nitrogen sources on organic acid excretion. Hobbs et al. (1992) maintained the nitrogen sources tested at 30mM, thus supplying varying amounts of initial nitrogen.

For the work presented in this section, initial nitrogen was varied at the simplest level to give 0.21, 0.42 or 0.63g·L\(^{-1}\). Exact balancing of both initial carbon and nitrogen would have been impractical while keeping the initial glucose constant. At this stage of the work, glucose was thought to be the major cause of acidification. The approach described above was suitable for studying production of acids by *S. lividans* during growth on glucose, and the subsidiary effects of nitrogen sources. Acid yield can be presented as a percentage of the consumed initial carbon source, enabling direct comparisons between media.

The results presented in this section are divided into four groups: 3.2.2.1, glucose with inorganic nitrogen sources (media 1-2 in Table 3.6); 3.2.2.2, glucose with organic nitrogen sources (media 3-7); 3.2.2.3, glucose with mixed nitrogen sources (media 8-11); and 3.2.2.4, media without glucose (media 12-19).

All experiments were repeated at least twice. Data presented for each medium is typical data taken from one experiment (unless otherwise stated). This is applicable to data also presented in Sections 3.4 to 3.6. Samples were taken in duplicate at each timepoint. Comparison of repeat data for all defined medium types (Section 3.2 and 3.4) showed that values varied on average by less than 2.9%. In all cases trends were the same, for example maximum biomass, deoxyribose and acid levels occurred at the same times in repeat cultures. Calculations of acid concentrations and carbon and nitrogen changes and percentages were corrected to allow for the volume of culture removed by sampling (approximately one fifth over the course of each 120h culture).
<table>
<thead>
<tr>
<th>No.</th>
<th>MEDIUM COMPOSITION (SMM base)</th>
<th>DESIGNATION</th>
<th>INITIAL C (g·L⁻¹)</th>
<th>INITIAL N (g·L⁻¹)</th>
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<tr>
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<td>glucose-nitrate</td>
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<td>aspartate-ammonium</td>
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<td>aspartate-nitrate</td>
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<td>1.05</td>
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*Table 3.6: Media used in the shake flask cultures described in Section 3.2.*
3.2.2 Results

3.2.2.1 Glucose with Inorganic Nitrogen Sources

When *S. lividans* TK24 was grown in SMM media containing glucose and ammonium, no organic acids were detected by HPLC in samples of filtered extracellular medium. Data for a culture in this medium is shown in Figures 3.3 and 3.4. Growth was poor and dry cell weight (DCW) reached levels of only 0.9g-L\(^{-1}\) at 48h of the 120h duration. Biomass was in the form of dispersed mycelia and some small visible pellets. DNA levels continued to increase until 54h, indicating that some replicatory growth continued after the point of maximum biomass. The fall in biomass after 48h may have been due to lysis of material as biomass was still accumulating. Only around 50% of the glucose supplied was utilised by the organism, with major consumption occurring in the period 30 to 48h (Figure 3.4). About 70% of the initial ammonium was utilised, also mainly in the first 48h (not shown). The pH of the culture did not change significantly from 6.5 (not shown).

In contrast, when ammonium salts were replaced with nitrate as the primary nitrogen source, *S. lividans* excreted large amounts of organic acids into the extracellular medium. These were identified by HPLC and enzymic assays (see Section 3.1) as pyruvate and \(\alpha\)-ketoglutarate. Growth data from a culture grown in glucose-nitrate medium (Figure 3.5) demonstrates that *S. lividans* also grew better when supplied with nitrate rather than ammonium. Biomass levels reached 2.1g-L\(^{-1}\) DCW at 72h, accompanied by the exhaustion of glucose at 78h. Biomass was observed to be an approximately equal mixture of pellets and dispersed mycelia. It can be seen from Figure 3.5 that during the period of rapid growth the increase in biomass was linear rather than exponential (confirmed using log data). This might be related to carbon or nitrogen limitation in this medium. It was likely that the medium was carbon-limited (refer to Section 3.7). Alternatively, linear growth could be related to limitations caused to mycelia by pelleting. The effects of pelleting are discussed in Sections 1.4.2 and 4.3. Linear growth was common to most shake flasks during this project, and is noted by Prosser & Tough (1991) to be common to *Streptomyces* cultures containing pelleted mycelia. The peak in biomass and end of true growth (shown by the end of deoxyribose increase) occurred later than in glucose-ammonium cultures. This could be explained by the elevated levels of biomass. However it can be seen from Figures 3.4 and 3.6 that the
Figure 3.3: Graph showing biomass (●) and deoxyribose (▼) changes in a culture grown in SMM containing 30mM glucose and 15mM ammonium salt.

Figure 3.4: Graph showing changes in carbon source, (glucose ■) during a culture grown in SMM containing 30mM glucose and 15mm ammonium salt. Ammonium not shown but present throughout.
rapid uptake of glucose was delayed in glucose-nitrate medium and did not occur until after 48h. This may indicate that ammonium was more readily utilised as a nitrogen source than nitrate in the early stages of growth of *S. lividans*. Ahmed *et al.* (1984) found that while nitrate supported better growth by *S. venezuelae* than ammonium, early growth was more rapid in glucose-ammonium medium than glucose-nitrate medium.

Organic acids were first detected in the medium at 30h (Figure 3.6). Levels increased during the rapid growth phase to reach maxima of 475.5mg·L⁻¹ pyruvate and 151.9mg·L⁻¹ α-ketoglutarate, both at 78h. Acid release was accompanied by a fall in pH from 6.5 to 5.0 (Figure 3.7). The pH fell most drastically during the accumulation of pyruvate. DNA analysis (Figure 3.5) showed that true replicatory growth ended at 72h, but it is evident that active metabolism continued into the stationary phase, as would be expected (Prosser & Tough, 1991), by the continued use of glucose to exhaustion at 78h and the formation of organic acids also to 78h.

After 78h the amounts of organic acids in the medium began to decrease, with an associated rise in pH. This observation suggests that the acids were being reassimilated by the organism, probably to support further ‘turnover’ metabolism, secondary metabolism and formation of storage materials occurring in the stationary phase. Neither acid was reused completely, although pyruvate was taken up more rapidly and more of this acid was reused than of α-ketoglutarate. The rise in pH towards the end of growth has been described as characteristic of acidifying *S. lividans* cultures (Wrigley-Jones *et al.*, 1993). During stationary phase the biomass levels fell by around a quarter. Foaming occurred around 120h, probably due to lysis of material (which is common in defined media; Bushell & Fryday, 1983).

Weight for weight, around three times more pyruvate was excreted than α-ketoglutarate. Of the total carbon consumed, a maximum of 8.1% passed to pyruvate and 2.6% to α-ketoglutarate (Figure 3.8). Since the acid maxima occurred at the same time it can be said that up to 10.7% of the carbon from glucose was converted to organic acids in this medium. Further carbon analysis (Section 2.1.10) showed that the biomass contained an average of 38.1% w/w carbon and that around 66% of the initial/consumed carbon from the medium was lost through respiratory activity to CO₂.

These results show that *S. lividans* produces organic acids which are commonly produced by a number of *Streptomyces* species during growth on glucose. In this strain, acid production is influenced by the nitrogen source present and is supported by nitrate but not ammonium. These results will be discussed in Section 4.
Figure 3.5: Graph showing biomass (●) and deoxyribose (▼) changes in a culture grown in SMM containing 30mM glucose and 30mM nitrate salt.

Figure 3.6: Graph showing changes in carbon source, glucose (■) and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in SMM containing 30mM glucose and 30mM nitrate salt. Nitrate not shown but present throughout.
Figure 3.7: Changes in pH during a culture grown in SMM containing 30mM glucose and 30mM nitrate salt. NB pH measurements were discrete, not continuous.

Figure 3.8: Graph showing decrease in total carbon (○) and proportion of carbon present in organic acids pyruvate (•) and α-ketoglutarate (△), expressed as a % of total carbon consumed during a culture grown in SMM containing 30mm glucose and 30mM nitrate.
3.2.2.2 Glucose with Organic Nitrogen Sources

For this set of shake flasks, inorganic nitrogen sources were replaced by amino acids as organic nitrogen sources. These may also act as secondary carbon sources. Five amino acids were chosen for being soluble in liquid media, readily utilised by *S. lividans* TK24, and representative of the methods by which amino acids are metabolised by bacteria. These are described in Table 3.7.

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>alanine</td>
<td>non-polar side chain; transaminated to pyruvate</td>
</tr>
<tr>
<td>glycine</td>
<td>non-polar side chain; converted ultimately to pyruvate via 2 enzymic steps</td>
</tr>
<tr>
<td>aspartate</td>
<td>carboxyl group side chain; transaminated to enter TCA cycle directly via oxaloacetate</td>
</tr>
<tr>
<td>glutamate</td>
<td>carboxyl group side chain; deaminated to enter TCA cycle directly via α-ketoglutarate</td>
</tr>
<tr>
<td>lysine</td>
<td>amino group side chain; converted ultimately to acetoacetyl CoA and acetyl CoA via at least 8 enzymic steps</td>
</tr>
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</table>

*Table 3.7: Amino acids used as organic nitrogen sources (details from Stryer, 1995).*

*S. lividans* TK24 grew well in glucose-alanine medium, achieving a dry cell weight of 2.8g·L⁻¹ at 72h (Figure 3.9). DNA analysis indicated that replicatory growth continued until 78h. These results demonstrate that certain amino acids are capable of supporting greater biomass accumulation than ammonium or nitrate salts [also observed for other streptomycetes by Ahmed *et al.* (1984) and Liao *et al.* (1995)]. Pyruvate and α-ketoglutarate were first detected in the medium at 24h (Figure 3.10). Unlike glucose-nitrate medium, the levels of the acids did not rise and fall at the same time. Pyruvate increased during rapid growth and peaked at a maximum of 328.5mg·L⁻¹ at 54h. Levels of this acid then decreased rapidly until it was completely reused by around 72h. As shown in Figure 3.10 this corresponds to the time when glucose and alanine were also exhausted from the medium and to the peak in biomass levels (Figure 3.9). α-Ketoglutarate increased at a slower rate until 54h and then rapidly until reaching a maximum of 415.0mg·L⁻¹ at 72h, corresponding to the peak in biomass. The pH fell to 5.0 at 54h (Figure 3.11). The lowest pH corresponded to the greatest levels of pyruvate. While pyruvate levels did not greatly exceed α-ketoglutarate levels as in glucose-nitrate
Figure 3.9: Graph showing biomass (●) and deoxyribose (←) changes in a culture grown in SMM containing 30mM glucose and 15mM alanine.

Figure 3.10: Graph showing changes in carbon and nitrogen sources, glucose (■) and alanine (←), and organic acids, pyruvate (●) and α-ketoglutarate (←) during a culture grown in SMM containing 30mM glucose and 15mM alanine.
Figure 3.11: Changes in pH during a culture grown in SMM containing 30mM glucose and 15mM alanine.

Figure 3.12: Graph showing decrease in total carbon (—□—) and proportion of carbon present in organic acids pyruvate (—●—) and α-ketoglutarate (—▲—), expressed as a % of total carbon consumed during a culture grown in SMM containing 30mM glucose and 15mM alanine.
medium, the fact that the pH fell more during pyruvate excretion suggests that this metabolite had a greater effect on medium pH. The pKa of pyruvate (2.39) is lower than α-ketoglutarate (2.47-4.68; Dawson et al., 1986). After 54h, the pH rose even though α-ketoglutarate continued to be excreted.

From Figure 3.12 it can be seen that the reuse of pyruvate occurred at an equivalently rapid rate to the utilisation of the remaining carbon sources between 54 and 72h. The reuse of ketoglutarate between 72 and 78h occurred at a similar rate. From these results it would appear that reassimilated pyruvate contributed to fuelling rapid growth and biomass accumulation by S. lividans TK24. It is possible that some α-ketoglutarate was also reused for this purpose until replicatory growth ceased at 78h. After 78h α-ketoglutarate levels fell slightly but this acid was not completely reused. In this period, biomass levels also fell by around 50%.

By weight, marginally more α-ketoglutarate was excreted than pyruvate. Of the consumed carbon, around 4.8% passed to pyruvate and 6.3% to α-ketoglutarate (Figure 3.12). As much as 11.1% of the consumed carbon could be converted into organic acids. It is also possible that some pyruvate was converted to α-ketoglutarate in the period 54-72h when pyruvate was reassimilated, hence the total amount of consumed carbon excreted as acids might be less.

The fermentation profiles for glucose-glycine cultures were similar to those from glucose-alanine cultures. S. lividans TK24 grew well but slowly to attain a maximum DCW of 2.4g.L⁻¹ at 96h (Figure 3.13). DNA increase also ceased at this time. A period of rapid growth occurred between 54 and 72h, during which the biomass doubled, and which corresponded to the rapid consumption of both glucose and glycine to exhaustion by 72 to 78h (Figure 3.14). In the same period, pyruvate levels increased and peaked at 294.1mg.L⁻¹ at 72h. Between 72 and 96h biomass levels increased slowly from 2.3 to 2.4g.L⁻¹, accompanying the rapid and complete reuse of pyruvate from the medium. Again, pyruvate reuse occurred at a rapid rate concurrent with the utilisation of the remaining carbon sources from 72 to 78h, after which pyruvate reuse was slower. α-Ketoglutarate levels increased slowly to a maximum of 197.2mg.L⁻¹ at 96h and decreased slightly by 120h. After 96h, biomass levels fell by about 10%. The pH fell during acid production to 5.5 at the time when extracellular pyruvate levels were the greatest (Figure 3.15). The pH rose during rapid pyruvate reuse but while α-ketoglutarate levels continued to increase.
Figure 3.13: Graph showing biomass (— ) and deoxyribose (— ) changes in a culture grown in SMM containing 30mM glucose and 15mM glycine.

Figure 3.14: Graph showing changes in carbon and nitrogen sources, glucose (— ), and glycine (— ), and organic acids, pyruvate (— ) and α-ketoglutarate (— ) during a culture grown in SMM containing 30mM glucose and 15mM glycine.
Figure 3.15: Changes in pH during a culture grown in SMM containing 30mM glucose and 15mM glycine.

Figure 3.16: Graph showing decrease in total carbon (■) and proportion of carbon present in organic acids pyruvate (●) and α-ketoglutarate (▲), expressed as a % of total carbon consumed during a culture grown in SMM containing 30mM glucose and 15mM glycine.
By weight, 50% more pyruvate was excreted than α-ketoglutarate. Of the consumed carbon, up to 5.0% was converted to excreted pyruvate and 3.4% to α-ketoglutarate (Figure 3.16). Maximum acid levels were less than in glucose-alanine cultures. Since comparable DCW levels were recorded, but glycine supplies less carbon than alanine, it is possible that carbon was more efficiently converted to biomass in this medium.

The fermentation profiles for cultures grown in glucose-aspartate and glucose-glutamate media were different from those cultures described above. In glucose-aspartate media, two distinct biomass peaks were seen (Figure 3.17). The first (2.9g·L⁻¹ DCW at 48h) corresponded to the exhaustion of the amino acid from the medium (Figure 3.18). The second (3.0g·L⁻¹ at 78h) corresponded to the exhaustion of glucose from the medium. It is unlikely that aspartate would be preferred as a carbon source to glucose, and the consecutive use may instead reflect its use as a nitrogen source and the elevated biomass levels obtained in this medium. Aspartate was clearly used at a faster rate than alanine or glycine and at an at least equal rate to glucose from 24 to 48h.

Pyruvate was excreted during the period in which glucose was rapidly utilised and peaked at 301.2mg·L⁻¹ at 72h. The rapid reuse of pyruvate began after glucose was exhausted. α-Ketoglutarate levels increased rapidly between 24 and 48h and then to a maximum of 932.1mg·L⁻¹ at 78h (Figure 3.18). The pH of the medium fell to 5.0 during acid accumulation until 78h (Figure 3.19). The pH continued to fall during α-ketoglutarate accumulation, possibly due to the elevated excretion of this metabolite compared to previous cultures. Although DNA analysis (Figure 3.17) shows that true growth also ceased at this point, the rapid reuse of both pyruvate and α-ketoglutarate after 78h suggests that very rapid metabolism continued into the stationary phase. Although α-ketoglutarate was not completely reused, it is possible that it would have been had it been excreted at lower levels by this culture (compare Figure 3.18 to Figures 3.10 and 3.14). Three times more α-ketoglutarate than pyruvate was excreted by weight. Of the consumed carbon, pyruvate accounted for a maximum of 4.4% and α-ketoglutarate for up to 13.8% (Figure 3.20). Increased availability of carbon from aspartate might explain the ability of the organism to attain both increased biomass levels and greater acid output in this medium.
Figure 3.17: Graph showing biomass (●) and deoxyribose (▲) changes in a culture grown in SMM containing 30mM glucose and 15mM aspartate.

Figure 3.18: Graph showing changes in carbon and nitrogen sources, glucose (■) and aspartate (▲), and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in SMM containing 30mM glucose and 15mM aspartate.
Figure 3.19: Changes in pH during a culture grown in SMM containing 30mM glucose and 15mM aspartate.

Figure 3.20: Graph showing decrease in total carbon (—□—) and proportion of carbon present in organic acids pyruvate (●—) and α-ketoglutarate (▲—), expressed as a % of the total carbon consumed during a culture grown in SMM containing 30mM glucose and 15mM aspartate.
With glucose-glutamate medium, the profiles were less clear but followed similar trends to glucose-aspartate cultures. Two biomass peaks were observed, the first (3.2g·L⁻¹ at 48h) coincident with glutamate exhaustion and the second (3.3g·L⁻¹ at 120h) occurring at glucose exhaustion (Figures 3.21 and 3.22). Pyruvate appeared at 24h, increased to a maximum of 303.8mg·L⁻¹ at 78h and was rapidly and completely reused by 120h perhaps again contributing to biomass accumulation. Although DNA analysis showed the end of true growth to be at 96h, both pyruvate reuse and α-ketoglutarate formation continued until 120h, again suggesting that very rapid metabolism continued into the stationary phase. α-Ketoglutarate levels peaked at 638.5mg·L⁻¹ at 120h. A sample at 144h showed that α-ketoglutarate levels fell after 120h. During acid excretion the pH fell to 5.0 and remained at a low value due to continued α-ketoglutarate production (Figure 3.23). A slight rise in pH corresponded to pyruvate reuse but during α-ketoglutarate excretion, and again indicated the greater influence of pyruvate on the medium pH.

By weight, twice as much α-ketoglutarate was produced by the organism. Of the total consumed carbon, around 4.0% passed to pyruvate and 8.8% to α-ketoglutarate (Figure 3.24). Both increased carbon supply from glutamate and relatively reduced acid production may have resulted in enhanced biomass yield although glucose was poorly used in these cultures.

*S. lividans* TK24 grew slowly in glucose-lysine medium and reached a maximum DCW of 2.0g·L⁻¹ at 120h (Figure 3.25). DNA levels continued to increase beyond the end of the culture. After a long lag period of around 72h, 50% of the lysine and almost all of the glucose was utilised (Figure 3.26); in total around 80% of the initial carbon was utilised over 120h. Pyruvate and α-ketoglutarate appeared at 24h as previously. Pyruvate levels reached 104.8mg·L⁻¹ at 96h, followed by rapid reassimilation almost to exhaustion by 120h. It was also in the period 96 to 120h that (i) the most rapid use of glucose and lysine occurred; (ii) DNA levels increased very rapidly; and (iii) a rapid increase in the level of α-ketoglutarate was observed, increasing to 238.1mg·L⁻¹ by 120h. The pH fell slightly during this culture and did not rise again (Figure 3.27) although most of the excreted pyruvate was reused. The pH may have been retained at a low level by the rapidly increasing α-ketoglutarate levels at the end of the culture and by the late metabolism of glucose. By weight, twice as much α-ketoglutarate was detected
Figure 3.21: Graph showing biomass (–•–) and deoxyribose (—▲—) changes in a culture grown in SMM containing 30mM glucose and 15mM glutamate.

Figure 3.22: Graph showing changes in carbon and nitrogen sources, glucose (–■–) and glutamate (—▲—), and organic acids, pyruvate (–●–) and α-ketoglutarate (—□—) during a culture grown in SMM containing 30mM glucose and 15mM glutamate.
Figure 3.23: Changes in pH during a culture grown in SMM containing 30mM glucose and 15mM glutamate.

Figure 3.24: Graph showing decrease in total carbon (—□—) and proportion of carbon present in organic acids pyruvate (—●—) and α-ketoglutarate (—▲—), expressed as a % of the total carbon consumed during a culture grown in SMM containing 30mM glucose and 15mM glutamate.
Figure 3.25: Graph showing biomass (●) and deoxyribose (▼) changes in a culture grown in SMM containing 30mM glucose and 15mM lysine.

Figure 3.26: Graph showing changes in carbon and nitrogen sources, glucose (■) and lysine (►), and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in SMM containing 30mM glucose and 15mM lysine.
Figure 3.27: Changes in pH during a culture grown in SMM containing 30mM glucose and 15mM lysine.

Figure 3.28: Graph showing decrease in total carbon (-) and proportion of carbon present in organic acids pyruvate (●) and α-ketoglutarate (▲), expressed as a % of the total carbon consumed during a culture grown in SMM containing 30mM glucose and 15mM lysine.
in the medium compared to pyruvate. At most, pyruvate accounted for 1.8% and α-ketoglutarate for 4.2% of the carbon consumed during the culture (Figure 3.28).

The results presented in this section demonstrate variations in the metabolism of different carbon and nitrogen sources by *S. lividans* TK24. Compared to glucose-nitrate media, cultures in glucose-amino acid media (excluding lysine) produced less pyruvate but more α-ketoglutarate. These maxima are summarised in Table 3.8 in Section 3.2.3.

In most media, the amount of pyruvate excreted was remarkably similar, considering the differing chemistry of the amino acids and the positions of entry of their carbon skeletons into the TCA cycle. Alanine and glycine enter ultimately at pyruvate, perhaps explaining why α-ketoglutarate levels were equal to or less than pyruvate levels in media containing these amino acids. Aspartate, glutamate and lysine enter the TCA cycle beyond pyruvate as shown in Table 3.7, which may be related to the enhanced α-ketoglutarate levels relative to pyruvate media containing these amino acids. These results will be discussed further in Section 4.

Whereas the inorganic nitrogen sources described in Section 3.2.2.1 were present in excess throughout, the organic nitrogen sources described in this section were mostly exhausted by the end of the rapid growth phase. This may suggest that the organism was nitrogen limited in the stationary phase, but it is thought that any turnover or secondary metabolism occurring then would be supported by internal nitrogen supplies. Biomass accumulation was obviously not prevented by nitrogen deficiency in glucose-aspartate and glucose-glutamate cultures. Nitrogen limitation may be related to the fall in DCW occurring towards the end of these cultures, although this could be due to partial lysis of mycelia or catabolism of internal storage materials. Nitrogen deficiency probably did not prevent the organism reusing α-ketoglutarate since this acid was also not reused in glucose-nitrate cultures when nitrogen was present in excess.

Final observations of note are that the morphological form of the biomass in all of the glucose-amino acid cultures was an approximately equal mixture of pellets and dispersed mycelia, except media containing lysine where material existed as large flakes. Carbon analysis showed that the average carbon content of the biomass was 40.2%, which is consistent with earlier observations; and that an average of 65.7% of the carbon consumed by each culture was lost *via* respiration to CO₂ (excluding glucose-lysine cultures at a loss of 38.2% of consumed carbon to CO₂).
3.2.2.3 Glucose with Mixed Nitrogen Sources

Following the results obtained using inorganic and organic nitrogen sources (3.2.2.1 and 3.2.2.2), a set of experiments was performed using media containing mixed nitrogen sources. In each case glucose was combined with one inorganic and one organic nitrogen source, supplying a total of 0.63g·L⁻¹ nitrogen but no more than a 36% increase in carbon relative to the original glucose-ammonium media, taken as standard.

Two amino acids were chosen for further study, alanine and aspartate. Both supported high biomass yield and significant acidification, and were representative of the overall differences observed between alanine/glycine and aspartate/glutamate as described in Section 3.2.2.2. Glycine and glutamate were not used since these amino acids gave low acidification and high pigmentation respectively. Lysine was not used further due to poor growth and low acid production by the organism when grown on this nitrogen source.

Consistent with earlier results, the addition of ammonium salts to defined media repressed the overproduction of organic acids. The growth profile of the organism varied with the amino acid supplied. In glucose-aspartate-ammonium medium \( S. lividans \) grew well and achieved a DCW of 3.3g·L⁻¹ at 54h (Figure 3.29). Analysis showed that glucose was exhausted by 48h and aspartate by 54h (Figure 3.30). Ammonium was present throughout although around 75% was consumed. Although ammonium was still available, the sharp drop in biomass after 54h and the failure of the culture to utilise all of the ammonium may indicate nutrient limitation. The rapid use of carbon and nitrogen sources began at around 30h, consistent with glucose-ammonium medium. Biomass was observed to be in the form of dispersed mycelia throughout.

By contrast, \( S. lividans \) grew very poorly in glucose-alanine-ammonium medium, achieving a DCW of 1.1g·L⁻¹ at 48h (Figure 3.31). Heavy pelleting of the biomass was observed. DNA analysis indicated that replicatory growth continued until 54h but with no further increase in biomass. HPLC analysis showed that 66% of the initial glucose was consumed rapidly in the period 0 to 54h, after which medium levels were constant at approximately 10mM (Figure 3.32). About 70% of the ammonium was utilised, but very little of the alanine (25% of the alanine was consumed by 120h). It appears that ammonium does not support high biomass accumulation by \( S. lividans \) whilst amino acids are better for this purpose. Perhaps in a mixed nitrogen source medium, excess ammonium competed with organic nitrogen sources and delayed their
Figure 3.29: Graph showing biomass (•) and deoxyribose (▼) changes in a culture grown in SMM containing 30mM glucose, 15mM aspartate and 15mM ammonium salt.

Figure 3.30: Graph showing changes in carbon and nitrogen sources, glucose (■) and aspartate (▼) during a culture grown in SMM containing 30mM glucose, 15mM aspartate and 15mM ammonium salt. Ammonium not shown but present throughout.
Figure 3.31: Graph showing biomass (••) and deoxyribose (▼▼) changes in a culture grown in SMM containing 30mM glucose, 15mM alanine and 15mM ammonium salt.

Figure 3.32: Graph showing changes in carbon and nitrogen sources, glucose (■■) and alanine (▼▼) during a culture in SMM containing 30mM glucose, 15mM alanine and 15mM ammonium salt. Ammonium not shown but present throughout.
uptake by the organism. This depended on the amino acid present, for example aspartate seemed to be more readily utilised by *S. lividans* in the presence of ammonium; whilst alanine was not used at all (compare Figures 3.3 to 3.31 and 3.4 to 3.32 where it can be seen that glucose-ammonium and glucose-alanine-ammonium cultures proceeded in an identical fashion, as if alanine was absent from the latter).

A very small amount of pyruvate was detected in the medium of glucose-alanine-ammonium cultures, usually at a single time (48h). HPLC, ‘spiking’ and enzymic assays confirmed this to be pyruvate at a maximum level of 81.0mg-L\(^{-1}\). Alanine uptake was greatest in the period 24-48h and may have resulted in ‘leakage’ from the cells of pyruvate involved in a mechanism of ammonium uptake *via* alanine dehydrogenase (Shapiro & Vining, 1983). The pH of media containing ammonium did not change significantly from 6.5 (data not shown).

Glucose-amino acid-nitrate media supported the excretion of organic acids. It was found that the acid profiles were very similar to glucose-nitrate media, perhaps indicating some sort of interference between the nitrogen sources.

In glucose-aspartate-nitrate medium, the biomass increased to a level of 2.6g-L\(^{-1}\) DCW at 72h, with DNA analysis showing that replicatory growth continued until 78h (Figure 3.33). Both glucose and aspartate were exhausted by 72h (Figure 3.34) and nitrate remained present throughout. Pyruvate and \(\alpha\)-ketoglutarate appeared in the medium at around 30h (Figure 3.34). Pyruvate levels increased rapidly and \(\alpha\)-ketoglutarate levels more slowly until the acids reached maximum concentrations of 725.6mg-L\(^{-1}\) and 151.9mg-L\(^{-1}\) respectively at 72h. By this time the pH had fallen from 6.5 to 5.0 (Figure 3.35). After 72h, acid concentrations decreased rapidly and both were completely reused by 120h. The pH rose to 7.0 in this phase.

Compared to glucose-nitrate medium, more pyruvate was excreted, perhaps due to the contribution of additional carbon from aspartate. Significantly, \(\alpha\)-ketoglutarate levels were identical (see Section 3.3 for the relationship of aspartate to acid production). By weight, five times more pyruvate was produced than \(\alpha\)-ketoglutarate. In respect of consumed carbon, around 9.7% passed to pyruvate and 2.0% to \(\alpha\)-ketoglutarate (Figure 3.36). Acid maxima occurred at the same time indicating that up to 11.7% of the consumed carbon sources was excreted as organic acids. This was comparable to glucose-nitrate medium. In contrast to the glucose-nitrate medium, both acids were completely reused from the glucose-aspartate-nitrate medium. This may
Figure 3.33: Graph showing biomass (—•—) and deoxyribose (—■—) changes during a culture grown in SMM containing 3mM glucose, 15mM aspartate and 30mM nitrate.

Figure 3.34: Graph showing changes in carbon and nitrogen sources, glucose (—■—) and aspartate (—∥—), and organic acids, pyruvate (—●—) and α-ketoglutarate (—△—) during a culture in SMM containing 30mM glucose, 15mM aspartate and 30mM nitrate. Nitrate not shown but present throughout.
Figure 3.35: Changes in pH during a culture grown in SMM containing 30mM glucose, 15mM aspartate and 30mM nitrate.

Figure 3.36: Graph showing decrease in total carbon (□) and proportion of carbon present in organic acids pyruvate (●) and α-ketoglutarate (▲), expressed as a % of the total carbon consumed during a culture grown in SMM containing 30mM glucose, 15mM aspartate and 30mM nitrate.
indicate greater stationary phase metabolic activity by cultures grown in the latter medium, and may also be related to the greater carbon input and the fact that nitrogen was not limiting after aspartate exhaustion. A greater amount of consumed carbon was respired to CO₂ (76.1% from carbon analysis), and the biomass was found to contain an average of 47.3% carbon.

*S. lividans* did not grow well in glucose-alanine-nitrate medium, which was strikingly similar to the observation with glucose-alanine-ammonium medium discussed previously. The maximum DCW recorded was 1.6g-L⁻¹ at 48h (Figure 3.37). HPLC analysis (Figure 3.38) showed the consumption profiles for glucose and alanine to be similar to those in glucose-alanine-ammonium medium (Figure 3.32). The major uptake of glucose occurred from 0 to 54h after which very little was utilised. In total about 50% was consumed. Very little alanine was utilised (about 27%) and nitrate was present throughout. Organic acids were excreted (Figure 3.38), with associated changes in pH (Figure 3.39). In contrast to all previous results, most of the acid excretion occurred after biomass accumulation had apparently ceased. Pyruvate reached a concentration of 490.5mg-L⁻¹ while α-ketoglutarate levels reached 109.6mg-L⁻¹, both at 72h. The maximal points occurred at the same time as other nitrate-containing media. Of the consumed carbon, around 11.6% passed to pyruvate and 2.6% to α-ketoglutarate (Figure 3.40). The total of these (14.2%) was greater than for previous nitrate-containing media although less carbon source was consumed. Since the biomass level was lower, it is possible that in this medium the metabolism of the culture was directed away from biomass formation towards acid overproduction. After 72h, little of the acid was reassimilated, probably due to the metabolic state of the culture and reduced need for carbon sources. Between 72 and 96h, most of the α-ketoglutarate and 25% of the pyruvate was reused. In this period the biomass level was stable although it fell before 72h and after 96h. In both glucose-amino acid-nitrate media, the biomass existed as a mixture of pellets and dispersed mycelia. The pH (Figure 3.39) fell to 5.0 at 72h but did not increase greatly afterwards, probably because of high levels of acids remaining in the medium.

These results demonstrate that *S. lividans* metabolism varied depending on the nitrogen source(s) present. Ammonium salts repressed or did not support copious acid excretion. Nitrate and amino acids did support acid production. There appeared to be no
Figure 3.37: Graph showing biomass (---) and deoxyribose (----) changes during a culture grown in SMM containing 30mM glucose, 15mM alanine and 30mM nitrate.

Figure 3.38: Graph showing changes in carbon and nitrogen sources, glucose (---) and alanine (----), and organic acids, pyruvate (---) and α-ketoglutarate (-----) during a culture in SMM containing 30mM glucose, 15mM alanine and 30mM nitrate. Nitrate not shown but present throughout.
Figure 3.39: Changes in pH during a culture grown in SMM containing 30mM glucose, 15mM alanine and 30mM nitrate.

Figure 3.40: Graph showing decrease in total carbon (—□—) and proportion of carbon present in organic acids pyruvate (—●—) and α-ketoglutarate (—▲—), expressed as a % of the total carbon consumed during a culture grown in SMM containing 30mM glucose, 15mM alanine and 30mM nitrate.
relation between acid excretion and whether the nitrogen source was readily or poorly utilised by *S. lividans*. This will be discussed further in Section 4.

In addition inorganic and organic nitrogen sources appeared to 'interact' when present together in media. When nitrate was present either as the sole nitrogen source or combined with an amino acid, pyruvate levels were increased and α-ketoglutarate levels decreased, with maxima occurring simultaneously, compared to media without nitrate. In glucose-amino acid media, pyruvate concentrations were lower and α-ketoglutarate concentrations higher, and the acids increased and decreased at different times. The differences may reflect preference by the organism for one nitrogen source over another, or some metabolic requirement for nitrogen in a particular form. This will also be discussed in Section 4.
3.2.2.4 Media Without Glucose

To investigate the presumed dependence of acid production on the presence of glucose, *S. lividans* TK24 was grown in a number of defined media lacking glucose. The media contained an amino acid as both carbon and nitrogen source, with or without an alternative primary nitrogen source. The aim was to establish if amino acids supported acid excretion, and if so whether ammonium and nitrate had supplementary effects as before (Sections 3.2.2.1-3.2.2.3).

The growth profiles of *S. lividans* TK24 in the eight media tested are illustrated in Figure 3.41. Growth was poor and the dry cell weight did not exceed 1.5g-L\(^{-1}\) in media containing only glycine, lysine or aspartate. It was expected from previous cultures that the biomass yield would be low on glycine or lysine, but the result from aspartate was unexpected. Growth was moderately better in alanine and glutamate media, achieving DCW maxima of 1.8 and 2.4 g-L\(^{-1}\) respectively. From Figure 3.42, it can be seen that less than 20% of the carbon source was utilised from glycine and lysine media and around 50% from aspartate medium. Alanine was exhausted by 120h and glutamate by 72h. In the latter medium, biomass levels remained remarkably stable after 72h, considering the severe nutrient limitation imposed on the culture.

The biomass yield improved in media containing an extra nitrogen source compared to those containing only an amino acid. Aspartate-nitrate and aspartate-ammonium cultures yielded dry weight maxima of around 1.8g-L\(^{-1}\) (Figure 3.41). The inorganic nitrogen source was present in excess during cultures in both media, while 40% and 16% respectively of the aspartate was consumed (Figure 3.42). Although less aspartate was consumed, higher biomass levels may reflect extra nitrogen availability.

Alanine-ammonium cultures yielded a maximum dry weight of 3.6g-L\(^{-1}\). Ammonium was exhausted by 78h and alanine virtually by 96h, coincident with the time of the peak in biomass. This result is in sharp contrast to the poor growth observed in glucose-alanine-ammonium and glucose-alanine-nitrate media, and may reflect differences in the metabolism of *S. lividans* when presented with differing combinations of substrates. All cultures exhibited pelleting, except alanine-ammonium cultures in which pelleting was conspicuously absent. Rapid growth of these cultures was found to be exponential rather than linear, which may also be related to lack of pelleting (see Section 3.2.2.1).
Figure 3.41: Graph showing biomass changes during cultures in eight SMM-based media lacking glucose but containing alanine (—■—), glycine (—●—), aspartate (—▲—), glutamate (—◆—), lysine (—▲—), aspartate and nitrate (—△—), aspartate and ammonium (—□—) or alanine and ammonium (—○—). Details are in the text.

Figure 3.42: Graph showing changes in carbon sources in eight SMM-based media lacking glucose. Symbols as Figure 3.41, ammonium and nitrate changes are not shown. Details are in the text.
To summarise, acids were not excreted by any cultures in media lacking glucose or a similar rapidly-used carbon source, and the pH of cultures did not change appreciably from 6.5 (not shown). Good biomass yields in the absence of acid excretion in alanine-ammonium and glucose-aspartate-ammonium media provide a promising basis for defined physiological studies and the development of media supporting high recombinant product titres. However, the effect of ammonium ions on other metabolic pathways would need to be considered and the biomass yield tended to be greater in complex media (Section 3.5).
### 3.2.3 Summary

The observations described in Section 3.2.2 are summarised in Table 3.8.

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>MAXIMUM DRY CELL WEIGHT (g\text{-}L^{-1})</th>
<th>MAXIMUM PYRUVATE (mg\text{-}L^{-1})</th>
<th>MAXIMUM α-KETOGLUTARATE (mg\text{-}L^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose-ammonium</td>
<td>0.9 (48h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>glucose-nitrate</td>
<td>2.1 (72h)</td>
<td>475.5 (78h)</td>
<td>151.9 (78h)</td>
</tr>
<tr>
<td>glucose-alanine</td>
<td>2.8 (72h)</td>
<td>328.5 (54h)</td>
<td>415.0 (72h)</td>
</tr>
<tr>
<td>glucose-glycine</td>
<td>2.4 (96h)</td>
<td>294.1 (72h)</td>
<td>197.2 (96h)</td>
</tr>
<tr>
<td>glucose-aspartate</td>
<td>3.0 (78h)</td>
<td>301.2 (72h)</td>
<td>932.1 (78h)</td>
</tr>
<tr>
<td>glucose-glutamate</td>
<td>3.3 (120h)</td>
<td>303.8 (78h)</td>
<td>638.5 (120h)</td>
</tr>
<tr>
<td>glucose-lysine</td>
<td>2.0 (120h)</td>
<td>104.8 (96h)</td>
<td>238.1 (120h)</td>
</tr>
<tr>
<td>glucose-aspartate-ammonium</td>
<td>3.3 (54h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>glucose-alanine-ammonium</td>
<td>1.1 (48h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>glucose-aspartate-nitrate</td>
<td>2.6 (72h)</td>
<td>725.6 (72h)</td>
<td>151.9 (72h)</td>
</tr>
<tr>
<td>glucose-alanine-nitrate</td>
<td>1.6 (48h)</td>
<td>490.5 (72h)</td>
<td>109.6 (72h)</td>
</tr>
<tr>
<td>alanine</td>
<td>1.8 (72h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>glycine</td>
<td>1.2 (72h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>aspartate</td>
<td>1.2 (120h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>glutamate</td>
<td>2.4 (72h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>lysine</td>
<td>0.7 (78h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>alanine-ammonium</td>
<td>3.6 (96h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>aspartate-ammonium</td>
<td>1.8 (120h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>aspartate-nitrate</td>
<td>1.9 (120h)</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

Table 3.8: Summary of observations of maximum excreted organic acid levels and maximum dry cell weight values for the media described in Section 3.2.2 and listed in Table 3.6. The times at which each of the maxima were recorded are shown in parentheses.
3.3 Shake Flask Cultures Using Radiolabelled Carbon and Nitrogen Sources

3.3.1 Objective

Radiolabelled substrates were added to shake flask cultures in order to estimate the contribution of carbon from carbon sources including glucose and amino acids to the organic acids produced by *S. lividans* TK24. Differences between the two amino acids selected for further investigation (alanine and aspartate) were also studied. There were five experiments, shown in Table 3.9.

To minimise handling and exposure, culture samples were assayed only by HPLC, with the usual prefiltering step used to provide material for DCW assays. After separation by HPLC, fractions containing peaks representing either individual medium components or organic acid products were measured for radioactivity as described in Section 2.1.11. It was found that in all cases the count (cpm) increase and decrease from sample to sample matched the rise and fall of compounds indicated by HPLC analysis. Deoxyribose and pH were not measured in these cultures. Each experiment was performed only once.

The DCW and HPLC data obtained was found to differ from that generated from repeats using unlabelled substrates (Section 3.2). With unlabelled substrates the average variation between all data points was less than 2.9%. With labelled substrates, HPLC data differed by on average 18.9% and DCW readings by on average 32.8% from unlabelled cultures. However, acid production trends were the same with maxima occurring at the same times as previous cultures (Section 3.2). The bulk of the carbon and nitrogen source consumption occurred within the same time span as before, with small amounts of substrates remaining for longer periods (details Section 3.3.2). Biomass trends varied slightly (details Section 3.3.2). It was thought that variations were due to the different culture conditions employed. To minimise the amount of labelled substrate required shake flasks contained 200mL rather than 500mL medium. It has been found that culture scale, conditions and ratio of media to flask size affected the performance of *S. lividans* TK24 (Susan Robinson, personal communication). For reasons of safety, cultures were grown in a different laboratory shaker to that described in Section 2.1.3, set at 200rpm but fixed at 30°C.
Other potential experimental error may have arisen due to the number of steps involved in the preparation of samples for radioactivity counting (filtering, washing, HPLC separation, fraction collection, transfer of fractions to counting vials). However, the cpm readings from fractions prepared from samples taken immediately after inoculation were compared with reference dilutions of labelled glucose and found to be accurate (for example the actual cpm in samples taken at 0h of growth from flasks containing labelled glucose were 18050, 21190 and 22708, compared to an estimated cpm of 21673 from a reference dilution of labelled glucose). Variation between data (labelled and unlabelled repeats) suggests that the data principally provides a qualitative picture of the fate of consumed carbon.

<table>
<thead>
<tr>
<th>MEDIUM COMPOSITION (SMM base)</th>
<th>DESIGNATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>30mM glucose</td>
<td>14C glucose-nitrate</td>
</tr>
<tr>
<td>30mM nitrate</td>
<td></td>
</tr>
<tr>
<td>0.33mCi\cdot L^{-1} D-[U-^{14}C]glucose</td>
<td></td>
</tr>
<tr>
<td>30mM glucose</td>
<td>14C glucose-alanine</td>
</tr>
<tr>
<td>15mM alanine</td>
<td></td>
</tr>
<tr>
<td>0.33mCi\cdot L^{-1} D-[U-^{14}C]glucose</td>
<td></td>
</tr>
<tr>
<td>30mM glucose</td>
<td>glucose-14C alanine</td>
</tr>
<tr>
<td>15mM alanine</td>
<td></td>
</tr>
<tr>
<td>0.25mCi\cdot L^{-1} L-[U-^{14}C]alanine</td>
<td></td>
</tr>
<tr>
<td>30mM glucose</td>
<td>14C glucose-aspartate</td>
</tr>
<tr>
<td>15mM aspartate</td>
<td></td>
</tr>
<tr>
<td>0.33mCi\cdot L^{-1} D-[U-^{14}C]glucose</td>
<td></td>
</tr>
<tr>
<td>30mM glucose</td>
<td>glucose-14C aspartate</td>
</tr>
<tr>
<td>15mM aspartate</td>
<td></td>
</tr>
<tr>
<td>0.25mCi\cdot L^{-1} L-[U-^{14}C]aspartate</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.9: Media used in the shake flask cultures described in Section 3.3.*
3.3.2 Results

3.3.2.1 Radiolabelled Glucose with Nitrate

When \( S. \text{lividans} \ \text{TK24} \) was grown in \(^{14}\text{C}\)glucose-nitrate medium, DCW and HPLC data were almost identical to that obtained from cultures grown in unlabelled medium (refer to Figures 3.5, 3.6 and 3.8). The maximum DCW recorded was \(2.0 \text{g}\cdot\text{L}^{-1} \) at 72h. Pyruvate and \(\alpha\)-ketoglutarate appeared at around 30h and reached concentrations of \(464.1 \text{mg}\cdot\text{L}^{-1} \) and \(151.9 \text{mg}\cdot\text{L}^{-1} \) respectively, both at 78h. Glucose was exhausted by 72h, which may be related to the cessation of biomass accumulation at this time. Nitrate was in excess throughout. Pyruvate and \(\alpha\)-ketoglutarate represented 8.1% and 2.6% of the input carbon respectively.

Figure 3.43 shows the decrease of labelled glucose in the medium represented as radioactive count. This profile was identical to that obtained from HPLC (see Figure 3.6). The rapid use of glucose from the medium began at 30h in this culture compared to the delay until 48h in unlabelled medium, perhaps reflecting differences in the culture conditions. From Figure 3.43 it can be seen that at certain times during growth the amount of added labelled carbon existing as pyruvate was up to 16% and as \(\alpha\)-ketoglutarate was up to 8%. Percentages are higher than total carbon values due to the contribution of unlabelled carbon to the excreted acids.

The amount of labelled carbon lost to \(\text{CO}_2 \) during the course of the culture was calculated by subtracting the amount remaining in the medium at 120h (9.2%), the amount remaining in the biomass at 120h (22.9%) and the amount remaining as dissolved \(\text{CO}_2 \) (0.05%) from the initial amount in cpm (100%). Approximately 67.9% of the labelled carbon was lost to \(\text{CO}_2 \) via respiration which is in reasonable agreement with the amount calculated from carbon analysis of unlabelled cultures (66%).
Figure 3.43: Graph showing the decrease in labelled carbon source, D-[U-^{14}C]glucose (—) and the proportion of labelled carbon passing into excreted organic acids pyruvate(→) and α-ketoglutarate (—) during a culture grown in SMM containing 30mM glucose and 30mM nitrate.
3.3.2.2 Radiolabelled Glucose with Amino Acids

The biomass yield of *S. lividans* TK24 in either \(^{14}\text{C}\)glucose-alanine medium (culture A) or glucose-\(^{14}\text{C}\)-alanine medium (culture B) was less compared to that in unlabelled glucose-alanine medium. This may have been due to the different culture conditions. The maximum DCW levels reached were 2.0 and 2.3g-L\(^{-1}\) respectively, both at 78h (Figure 3.44). The utilisation of alanine and glucose varied (Figure 3.45) although the majority of each substrate was consumed by 72h, equivalent to the point of exhaustion previously observed (Figure 3.10).

The levels of excreted pyruvate and \(\alpha\)-ketoglutarate also varied from the original repeats, although maxima occurred at the same times (Figure 3.45). More pyruvate was produced by culture A but less by culture B, both relative to unlabelled medium. Part of this may be due to the higher initial glucose concentration in medium A than medium B (32.75mM vs. 28.85mM). The maximum pyruvate concentrations were 332.9mg-L\(^{-1}\) and 246.6mg-L\(^{-1}\) respectively, both at 54h. Pyruvate was not reused as rapidly as in unlabelled cultures, although between 60 and 80% was reassimilated in the period 48 to 78h before the peak in biomass levels. A similar amount of \(\alpha\)-ketoglutarate was detected in all glucose-alanine cultures, with maxima of 330.2mg-L\(^{-1}\) and 301.1mg-L\(^{-1}\) in cultures A and B respectively, both at 72h. The reuse of this acid was almost identical to unlabelled medium. From the HPLC data, pyruvate represented between 3.8 and 4.8% and \(\alpha\)-ketoglutarate 4.6 to 4.8% of the total consumed carbon in the pair of cultures.

Figure 3.46 shows that the consumption of labelled carbon occurred at the same rate in the two cultures from 48h onwards, to exhaustion of both at about the same time. This is an interesting observation, not apparent from the profiles generated from the HPLC data (Figure 3.45). The passage of labelled carbon into pyruvate initially occurred at different rates in the two cultures. After 48h labelled carbon passed into excreted pyruvate and was then reassimilated at parallel rates in the cultures for at least 24h. The conversion of labelled carbon to \(\alpha\)-ketoglutarate occurred at different rates until 78h. Notably, at the point when labelled pyruvate fell to low levels, labelled \(\alpha\)-ketoglutarate also began to be consumed at parallel rates by the two cultures. The similar requirement for pyruvate and \(\alpha\)-ketoglutarate by the two cultures between 54 and 120h may be due to nutrient limitation in this period.

Labelled carbon from both substrates was converted to organic acids. More carbon from alanine passed into both acids (27% in total) compared to carbon from glucose (15% in total). It was known by this stage that amino acids influenced organic
Figure 3.44: Graph showing biomass (●○-) changes during cultures grown in SMM media containing 30mM glucose and 15mM alanine, with added D-[U-14C]glucose (culture A, solid symbols) and L-[U-14C]alanine (culture B, hollow symbols).

Figure 3.45: Graph showing changes in carbon and nitrogen sources, glucose (●○-) and alanine (■▲▲), and organic acids, pyruvate (●○-) and α-ketoglutarate (■▲▲) during cultures grown in SMM media containing 30mM glucose and 15mM alanine, with added D-[U-14C]glucose (culture A, solid symbols) and L-[U-14C]alanine (culture B, hollow symbols).
acid excretion but it had been assumed that glucose was the major supplier of carbon to organic acids since glucose was the primary/preferred carbon source and alanine the secondary carbon source. Alternatively, it was thought that pyruvate may be derived from glucose and α-ketoglutarate from alanine since carbon skeletons of deaminated amino acids are ultimately channelled into the TCA cycle (albeit via pyruvate in some cases). A breakdown of the data indeed showed that more carbon passed from glucose to pyruvate (9%) than α-ketoglutarate (6%); and more carbon passed from alanine to α-ketoglutarate (16%) than pyruvate (11%).

The amount of labelled carbon lost to CO₂ was calculated as 70% for culture A and 72% for culture B. This is comparable to the unlabelled repeats (69%). Slight increases may reflect the reduced yield of biomass and reduced levels of excreted acids in the radiolabelled relative to unlabelled cultures.

Growth of S. lividans TK24 in ¹⁴Cglucose-aspartate medium (culture C) and glucose-¹⁴Caspartate medium (culture D) was again less compared to unlabelled media. The maximum DCW yields were 2.5 and 1.8g⁻¹L⁻¹ respectively (Figure 3.47). The biomass profile of culture C was similar to the repeats in glucose-aspartate medium with two peaks at 48 and 78h, corresponding to the almost complete use of aspartate and glucose respectively, although glucose was not exhausted until 96h in this culture (Figure 3.48). The profile of culture D was less similar although the two highest biomass peaks (Figure 3.47) again corresponded to the near exhaustion of aspartate and then glucose.

The acid production profile (Figure 3.48) closely resembled that of the unlabelled medium (Figure 3.18). Similar maximum acid levels were recorded for all cultures. Pyruvate reached maximum levels of 286.2mg⁻¹L⁻¹ and 335.5mg⁻¹L⁻¹ in cultures C and D respectively, both at 72h; while α-ketoglutarate reached concentrations of 939.4mg⁻¹L⁻¹ and 872.2mg⁻¹L⁻¹ at 78h. The rates of reuse were comparable to unlabelled medium, with pyruvate completely consumed by 96h in both flasks. Elevated pyruvate levels in culture D may be related to lower biomass and α-ketoglutarate production in this culture. From HPLC data, between 4.8 and 5.4% of the total consumed carbon was converted to pyruvate, with as much as 15.7 or 13.8% passing to α-ketoglutarate in cultures C and D respectively.
Figure 3.46: Graph showing the decrease in labelled carbon sources, D-[U-14C]glucose (culture A, ■ and solid symbols) and L-[U-14C]alanine (culture B, □ and hollow symbols) and the proportion of labelled carbon passing into excreted organic acids pyruvate (●○●) and α-ketoglutarate (△△△) during cultures grown in SMM containing 30mM glucose and 15mM alanine.

Figure 3.47: Graph showing biomass (●○●) changes during cultures grown in SMM media containing 30mM glucose and 15mM aspartate, with added D-[U-14C]glucose (culture C, solid symbols) and L-[U-14C]aspartate (culture D, hollow symbols).
Figure 3.48: Graph showing changes in carbon and nitrogen sources, glucose (■ □) and aspartate (▼ ▼), and organic acids, pyruvate (○ ○) and α-ketoglutarate (▲ ▲) during cultures grown in SMM media containing 30mM glucose and 15mM aspartate, with added D-[U-14C]glucose (culture C, solid symbols) and L-[U-14C]aspartate (culture D, hollow symbols).

Figure 3.49: Graph showing the decrease in labelled carbon sources, D-[U-14C]glucose (culture C, ■ ■ and solid symbols) and L-[U-14C]aspartate (culture D, ▼ ▼ and hollow symbols) and the proportion of labelled carbon passing into excreted organic acids pyruvate (○ ○) and α-ketoglutarate (▲ ▲) during cultures grown in SMM containing 30mM glucose and 15mM aspartate.
As shown in Figure 3.49, after 24h labelled carbon from the two substrates was consumed at different rates, in keeping with the exhaustion of the substrates at different times and the corresponding biomass peaks.

Analysis of radioactivity in sample fractions showed that considerably more labelled carbon passed into organic acids from glucose than aspartate. This was in contrast to the results obtained from cultures A and B. The total amount of labelled carbon from glucose passing to excreted acids was similar to glucose-alanine cultures (21%), with more passing to pyruvate (11%) than α-ketoglutarate (10%). Very little labelled carbon passed from aspartate to excreted acids (4.5%), which was not expected considering the greater total acid release in this medium compared to glucose-alanine medium. However more labelled carbon from aspartate passed to α-ketoglutarate (3%) than pyruvate (1.5%). It is possible that more carbon passed in total from alanine to acids since alanine carbon skeletons enter the TCA cycle at pyruvate, whereas aspartate skeletons enter the TCA cycle directly at oxaloacetate, which is beyond both pyruvate and α-ketoglutarate in the metabolic pathway (Stryer, 1995).

The amounts of labelled carbon lost to CO₂ from cultures C and D were calculated as 73% and 72% respectively. These were greater than from unlabelled medium (65%) and may reflect the reduced biomass yield and slight acid reduction in the labelled cultures.

These results indicate that carbon from a number of potential carbon sources is metabolised by S. lividans TK24 into excess organic acids which are then excreted. The specific conclusions from the experiments presented in Sections 3.2 and 3.3 were that (i) acids were not derived solely from glucose, but acid excretion did require the presence of glucose since amino acids alone did not support acidification even when in excess; (ii) excess acids were formed from carbon derived from any carbon source, but pyruvate stemmed mainly from glucose and α-ketoglutarate from amino acids; and (iii) the contribution of carbon from each amino acid varied with the specific type, possibly related to the final point of entry of the deaminated carbon skeletons into the TCA cycle. These results are discussed further in Section 4. The effects of alternative primary carbon sources and complex medium constituents are presented in the following sections.
3.3.3 Summary

The observations described in Section 3.3.2 are summarised in Table 3.10. A summary of previous results can be found in Table 3.8.

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>MAXIMUM DRY CELL WEIGHT (g·L⁻¹)</th>
<th>MAXIMUM PYRUVATE (mg·L⁻¹)</th>
<th>CONTRIBUTION OF LABELLED SOURCE TO PYRUVATE</th>
<th>MAXIMUM α-KETOGLUT (mg·L⁻¹)</th>
<th>CONTRIBUTION OF LABELLED SOURCE TO α-KETOGLUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹⁴C glucose-nitrate</td>
<td>2.0 (72h)</td>
<td>464.1 (78h)</td>
<td>16%</td>
<td>151.9 (78h)</td>
<td>8%</td>
</tr>
<tr>
<td>¹⁴C glucose-alanine</td>
<td>2.0 (78h)</td>
<td>332.9 (54h)</td>
<td>9%</td>
<td>330.2 (72h)</td>
<td>6%</td>
</tr>
<tr>
<td>glucose-alanine</td>
<td>2.3 (78h)</td>
<td>246.6 (54h)</td>
<td>11%</td>
<td>301.0 (72h)</td>
<td>16%</td>
</tr>
<tr>
<td>¹⁴C glucose-aspartate</td>
<td>2.5 (48h)</td>
<td>286.2 (72h)</td>
<td>11%</td>
<td>939.4 (78h)</td>
<td>10%</td>
</tr>
<tr>
<td>glucose-aspartate</td>
<td>1.8 (72h)</td>
<td>335.5 (72h)</td>
<td>1.5%</td>
<td>872.2 (78h)</td>
<td>3%</td>
</tr>
</tbody>
</table>

Table 3.10: Summary of results reported in Section 3.3.2. Data shown are maximum recorded values with times in parentheses.
3.4 Shake Flask Cultures Using SMM Containing Alternative Carbon Sources and Defined Nitrogen Sources

3.4.1 Objective

This set of cultures was used to assess the differences in acid production by *S. lividans* TK24 when alternative carbon sources to glucose were used. It was established previously that glucose was a major source of the carbon converted into excess acids, depending on the nitrogen source supplied.

Four alternative carbon sources were chosen: starch, maltose, fructose and glycerol. Each of these carbon sources passes directly into the glycolytic pathway at a point above pyruvate (see Figure 1.1). Starch (a polymer of glucose) is broken down to glucose and maltose *via* the action of amylase. Maltose (a dimer of glucose) may be formed as a product of starch catabolism and is converted to glucose by maltase enzymes before entering central metabolism. Fructose (an isomer of glucose) is converted to fructose-6-phosphate by fructokinase. Glycerol (the primary carbon source in MEP medium; Wrigley-Jones, 1991) enters glycolysis at glyceraldehyde-3-phosphate *via* the action of (de)hydrogenase and kinase enzymes.

Dekleva & Strohl (1987) found that *S. peucetius* cultures spontaneously acidified in defined glucose-nitrate and fructose-nitrate media; whereas cultures grown in nitrate-based media with starch, maltose and dextrin did not acidify. During the course of this project it was found that *S. lividans* TK24 did not grow well on sucrose, or dextrin (the DCW never exceeded 1.0g L⁻¹ and no organic acids were produced), hence these carbon sources were not tested further. The medium combinations used are shown in Table 3.11.

As for previous experiments, each combination was repeated at least twice and the average difference between all data points was less than 2.9%. Each carbon source was initially tested with nitrate as the sole nitrogen source (media 1-4). This was the simplest combination known to support acidification with glucose as the carbon source (Section 3.2.2.1). The carbon source supporting the highest acid excretion was combined with amino acids (media 5-6). Based on the results obtained from this, each other carbon source was tested in combination with amino acids (media 7-9).
Ammonium was not tested since this nitrogen source was known to suppress acid excretion in defined media.

Starch concentrations were calculated from HPLC data. It had not been expected that HPLC would separate starch due to its polymeric nature. However, extensive testing showed that this compound was separated reproducibly and accurately, allowing quantitation as % w/v against a standard curve (Section 3.1). This may be due to the use of soluble starch rather than potato starch in media. According to the manufacturers, soluble starch contains shorter chain length polymers, while potato starch contains longer chain lengths. HPLC calculations were confirmed by the use of carbon analysis (Section 2.1.10) on samples of starch-media. In all media starch was exhausted before obvious lysis of material (indicated by a fall in biomass and light foaming in shake flasks) which would have released carbon-containing materials and complicated starch measurement by carbon analysis.

<table>
<thead>
<tr>
<th>No.</th>
<th>MEDIUM COMPOSITION (SMM base)</th>
<th>DESIGNATION</th>
<th>INITIAL C (g L⁻¹)</th>
<th>INITIAL N (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5% w/v starch 30mM nitrate</td>
<td>starch-nitrate</td>
<td>2.03</td>
<td>0.42</td>
</tr>
<tr>
<td>2</td>
<td>15mm maltose 30mM nitrate</td>
<td>maltose-nitrate</td>
<td>2.00</td>
<td>0.42</td>
</tr>
<tr>
<td>3</td>
<td>60mm glycerol 30mm nitrate</td>
<td>glycerol-nitrate</td>
<td>2.16</td>
<td>0.42</td>
</tr>
<tr>
<td>4</td>
<td>30mM fructose 30mm nitrate</td>
<td>fructose-nitrate</td>
<td>2.00</td>
<td>0.42</td>
</tr>
<tr>
<td>5</td>
<td>0.5% w/v starch 15mM aspartate</td>
<td>starch-aspartate</td>
<td>2.88</td>
<td>0.21</td>
</tr>
<tr>
<td>6</td>
<td>0.5% w/v starch 15mm alanine</td>
<td>starch-alanine</td>
<td>2.58</td>
<td>0.21</td>
</tr>
<tr>
<td>7</td>
<td>15mM maltose 15mM aspartate</td>
<td>maltose-aspartate</td>
<td>2.88</td>
<td>0.21</td>
</tr>
<tr>
<td>8</td>
<td>60mm glycerol 15mM aspartate</td>
<td>glycerol-aspartate</td>
<td>2.72</td>
<td>0.21</td>
</tr>
<tr>
<td>9</td>
<td>30mM fructose 15mM aspartate</td>
<td>fructose-aspartate</td>
<td>2.72</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Table 3.11: Media used in the shake flask cultures described in Section 3.4.
3.4.2 Results

3.4.2.1 Alternative Carbon Sources with Nitrate

*S. lividans* TK24 grew well in starch-nitrate medium and produced biomass levels of up to 2.8g·L\(^{-1}\) DCW at 96h (Figure 3.50), which was greater than in glucose-nitrate medium (2.1g·L\(^{-1}\)). DNA analysis indicated that replicatory growth would have continued beyond 120h and the end of biomass accumulation probably reflects the exhaustion of starch at 72h (Figure 3.51). Nitrate was present throughout. Both pyruvate and \(\alpha\)-ketoglutarate appeared in the medium at around 30h but neither increased to the levels recorded for glucose-nitrate medium. Pyruvate reached a maximum concentration of 23.8mg·L\(^{-1}\) at 54h and \(\alpha\)-ketoglutarate a maximum of 57.0mg·L\(^{-1}\) at 72h. Acid production was accompanied by a slight fall in pH from 6.5 to 6.0, which was not as significant as in earlier cultures, although after 96h the pH rose sharply to 7.5 (Figure 3.52). The lowest pH corresponded to the highest acid levels. Had the culture continued beyond 120h, this high pH may have affected the organism. Biomass levels fell by 20% in the period of greatest pH increase, although the rise in pH accompanied the reuse of acids from the medium. Pyruvate was completely reused by 96h and may have been utilised to supply carbon during biomass accumulation up to 96h. \(\alpha\)-Ketoglutarate was virtually completely reused by the end of the culture. Since \(\alpha\)-ketoglutarate was not exhausted despite being excreted at only low levels, this suggests that this compound is not readily utilised by *S. lividans* as a potential carbon source in the way that pyruvate appears to be in defined media.

There were three significant differences between acid production in starch-nitrate and glucose-nitrate cultures. A comparable amount of carbon was consumed by both cultures (around 2g·L\(^{-1}\)). First, acid production was lower. Maximum pyruvate and \(\alpha\)-ketoglutarate levels represented just 0.5% and 1.0% respectively of the consumed carbon in starch-nitrate medium (Figure 3.53), compared to proportions of 8.1% and 2.6% in glucose-nitrate medium. Second, the acid maxima occurred at different times, whereas all glucose media containing nitrate exhibited simultaneous acid maxima (Sections 3.2.2.1 and 3.2.2.3). Third, by weight, almost twice as much carbon was excreted as \(\alpha\)-ketoglutarate, perhaps reflecting the extended period of production of this acid relative to pyruvate in starch-nitrate compared to glucose-nitrate medium.
Figure 3.50: Graph showing biomass (●) and deoxyribose (▲) changes during a culture grown in SMM containing 0.5% w/v starch and 30mM nitrate.

Figure 3.51: Graph showing changes in carbon source, starch (■), and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in SMM containing 0.5% w/v starch and 30mM nitrate. Nitrate not shown but present throughout.
Figure 3.52: Changes in pH during a culture grown in SMM containing 0.5% w/v starch and 30mM nitrate.

Figure 3.53: Graph showing decrease in total carbon ( — ) and proportion of carbon present in organic acids, pyruvate ( — ) and α-ketoglutarate ( — ), expressed as a % of the total carbon consumed during a culture grown in SMM containing 0.5% w/v starch and 30mM nitrate.
*S. lividans* grew moderately well but slowly in maltose-nitrate medium (Figure 3.54). The maximum DCW recorded was 2.7g·L⁻¹ at 120h, which was also an improvement on glucose-nitrate medium. DNA analysis indicated that replicatory growth continued beyond 120h (data not shown). Very little maltose was used in the initial stages of culture, levels falling by only around 6% in the first 72h (Figure 3.55). After 72h the carbon source was consumed more rapidly until around 75% had been used in total. This period of rapid maltose utilisation was accompanied by the appearance of a very small amount of pyruvate in the medium (10.8mg·L⁻¹) at 96h (not shown), corresponding to 0.07% of the total consumed carbon. No pyruvate was detected at other times during the culture, and no α-ketoglutarate was detected at all. The pH fell to 6.0 by 96h and rose rapidly to 7.0 by 120h (data not shown). Nitrate remained present throughout.

The biomass yields of *S. lividans* TK24 in glycerol-nitrate and fructose-nitrate media (Figure 3.54) did not exceed those of glucose-nitrate cultures, although glycerol was completely consumed (Figure 3.55). Both grew slowly and DNA analysis showed replicatory growth to continue beyond 120h (data not shown). Fructose-containing cultures grew steadily to a maximum DCW of 1.6g·L⁻¹ at 120h, but little fructose was utilised during the culture (Figures 3.54 and 3.55). Glycerol cultures grew slowly until 72h with the consumption of around 20% of the glycerol. Between 72 and 120h, the biomass doubled to a maximum dry weight of 2.0g·L⁻¹ and the remainder of the glycerol was utilised. Nitrate was present throughout cultures in both media. No acids were detected and the pH did not change from 6.5 (data not shown). The biomass in all cultures described in this section was in the form of finely dispersed mycelia.

The results demonstrated that organic acid excretion was not as copious when *S. lividans* TK24 was grown in defined media containing nitrate and an alternative carbon source to glucose. Those cultures excreting detectable amounts of acids were also those containing carbon sources which are metabolised directly to glucose (starch and maltose). The starch used was found to contain 0.2% reducing sugars in the form of glucose (manufacturer's notes), corresponding to 0.01g·L⁻¹ in the media described in this section. It is possible that this small amount of glucose induced acid production, causing higher acid production in starch-based media compared to maltose media. Maltose as supplied did not appear to contain glucose.
Figure 3.54: Graph showing biomass changes during three cultures in SMM containing maltose and nitrate (-- ■ --); glycerol and nitrate (-- ● --); or fructose and nitrate (-- △ --). Details are given in the text.

Figure 3.55: Graph showing changes in carbon sources during three cultures in SMM containing maltose (-- ■ --) and nitrate (not shown); glycerol (-- ● --) and nitrate (not shown); fructose (-- △ --) and nitrate (not shown). Details are in the text. Nitrate was present throughout all cultures.
3.4.2.2 Starch with Organic Nitrogen Sources

Following the results obtained previously by replacing inorganic with organic nitrogen sources (Section 3.2.2.2), *S. lividans* TK24 was grown in media containing starch and the amino acids chosen previously for further use, alanine and aspartate. Starch was chosen since it supported the highest acid production after glucose when combined with nitrate.

The growth of *S. lividans* TK24 in starch-aspartate medium is shown in Figure 3.56. Biomass levels rose to a maximum of 2.9g·L\(^{-1}\) DCW at 72h, which was confirmed to be the end of replicatory growth by DNA analysis. The biomass yield was similar to starch-nitrate cultures even though two potential carbon sources were available in starch-aspartate medium and both were exhausted during the culture. This occurred simultaneously at 54h (Figure 3.57), again showing a significant difference between starch-nitrate and glucose-nitrate cultures. In glucose-aspartate media, two biomass peaks were observed, corresponding to the consecutive exhaustion of aspartate and glucose (Figures 3.17 and 3.18). In starch-aspartate media there was a single biomass peak. Apparently starch was consumed more rapidly than glucose although the initial carbon supplied by these two carbohydrates was equivalent (2.19 and 2.04g·L\(^{-1}\) respectively).

Larger amounts of pyruvate and α-ketoglutarate were produced by starch-aspartate cultures (Figure 3.57) compared to starch-nitrate cultures, accompanied by a greater drop in pH from 6.5 to 5.0 by 78h (Figure 3.58). The maximum level of pyruvate recorded was 399.8mg·L\(^{-1}\) at 72h. Pyruvate was rapidly consumed and exhausted by 96h, suggesting that active metabolism again continued into the stationary phase. Pyruvate did not appear to contribute greatly to the maximal biomass accumulation in the distinct way that it did in glucose-amino acid cultures. Virtually all of the α-ketoglutarate produced (maximum 707.1mg·L\(^{-1}\) at 78h) was excreted after carbon source exhaustion. At least 30% of the maximum amount was excreted in the period 72-78h, also indicating very rapid metabolism after the end of replicatory growth. Almost no α-ketoglutarate was reused in the period when pyruvate was being rapidly reassimilated, suggesting that pyruvate may be preferred as a carbon source to α-ketoglutarate in some cultures. The pH was lowest when both pyruvate and α-ketoglutarate levels were high (Figure 3.58). The pH rose when pyruvate was reused but while α-ketoglutarate levels
Figure 3.56: Graph showing changes in biomass (■) and deoxyribose (▲) during a culture grown in SMM containing 0.5% w/v starch and 15mM aspartate.

Figure 3.57: Graph showing changes in carbon and nitrogen sources, starch (■) and aspartate (▲), and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in SMM containing 0.5% w/v starch and 15mM aspartate.
Figure 3.58: Changes in pH during a culture grown in SMM containing 0.5% w/v starch and 15mM aspartate.

Figure 3.59: Graph showing decrease in total carbon (–○–) and proportion of carbon present in organic acids pyruvate (–●–) and α-ketoglutarate (–▲–) expressed as a % of the total carbon consumed during a culture grown in SMM containing 0.5% w/v starch and 15mM aspartate.
remained high. Between 96 and 120h, α-ketoglutarate levels fell but the pH did not change, indicating again the greater effect of pyruvate on pH.

From Figure 3.59 it can be seen that of the consumed carbon, up to 5.6% passed to pyruvate and up to 10% to α-ketoglutarate. These values are lower by around 3% in total compared to glucose-aspartate cultures. By weight, around 1.8 times more α-ketoglutarate than pyruvate was excreted, which was in keeping with previous aspartate media.

*S. lividans* TK24 grew well in starch-alanine medium, achieving biomass levels of up to 3.0g L⁻¹ DCW at 78h while true replicatory growth continued until 96h (Figure 3.60). Substrate and metabolite profiles (Figure 3.61) were very similar to equivalent glucose-alanine cultures (Figure 3.10). Both carbon sources were exhausted by 72h (as before), explaining the failure of the culture to accumulate biomass further after 78h. Starch was initially used at a faster rate (around 33% was used by 30h, compared to the consumption of less than 1% glucose in the first 30h of glucose-alanine cultures) although the maximum biomass levels were comparable (3.0 and 2.8g L⁻¹).

Less of each acid was produced by these cultures (compared to glucose-alanine cultures) although the concentration of α-ketoglutarate was again slightly greater than pyruvate. Pyruvate levels reached 280.0mg L⁻¹ at 54h, with a fall in pH from 6.5 to 5.0 (Figure 3.62). Pyruvate was reused rapidly and was virtually exhausted by the biomass peak at 78h. It appeared that pyruvate carbon was consumed at a rapid rate with the remaining substrate carbon in the period until 72h (Figure 3.61), as with glucose-alanine cultures.

The peak in α-ketoglutarate concentrations was 298.0mg L⁻¹ at 78h, and was later than in glucose-alanine cultures. This may be related to the extended duration of biomass formation compared to glucose-alanine cultures. Approximately 50% of the α-ketoglutarate was reused after 78h with an associated rise in pH to 6.0. In relation to the consumed carbon, up to 4.3% was converted to pyruvate and 4.7% to α-ketoglutarate, a maximum of around 9% which was lower by 2% than glucose-alanine medium (Figure 3.63). It should be noted that the biomass existed as a mixture of pellets and dispersed mycelia in both starch-amino acid media.

These results show that when amino acids are added to media containing alternative carbon sources which do not support great acid formation when combined
Figure 3.60: Graph showing changes in biomass (●) and deoxyribose (▲) during a culture grown in SMM containing 0.5% w/v starch and 15mM alanine.

Figure 3.61: Graph showing changes in carbon and nitrogen sources, starch (■) and alanine (□), and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in SMM containing 0.5% w/v starch and 15mM alanine.
Figure 3.62: Changes in pH during a culture grown in SMM containing 0.5% w/v starch and 15mM alanine.

Figure 3.63: Graph showing decrease in total carbon (——) and proportion of carbon present in organic acids pyruvate (●) and α-ketoglutarate (▲) expressed as a % of the total carbon consumed during a culture grown in SMM containing 0.5% w/v starch and 15mM alanine.
with inorganic nitrogen sources, there is a large increase in acid output by *S. lividans*. Starch-amino acid cultures produced up to 95% more acids than starch-nitrate cultures. Also in starch-amino acid media, the maximum acid concentrations were comparable to those recorded in glucose-amino acid media. It can be concluded that starch is a no better carbon source than glucose when attempting to avoid organic acid excretion by *S. lividans* TK24 when amino acids are present, although acid excretion was reduced in media containing nitrate.
3.4.2.3 Maltose, Glycerol and Fructose with Organic Nitrogen Sources

Following the results presented in Section 3.4.2.2, *S. lividans* was grown in media containing the remaining alternative carbon sources combined with aspartate, to establish if acid production increased in these media relative to those containing nitrate. It was particularly important to establish whether the presence of the amino acid increased acid production in maltose and glycerol media since these carbon sources were the major types in the complex MEP medium used as part of this project.

*S. lividans* TK24 grew less well in maltose-aspartate medium than maltose-nitrate medium, reaching a maximum dry weight of 2.3g·L⁻¹ (Figure 3.64) compared to 2.7g·L⁻¹. Around 50% of the maltose was consumed and aspartate was exhausted by 78h (Figure 3.65). Up to 78h, less than 4% of the maltose was taken up from the medium and only 23% more was consumed by the biomass peak at 96h. It would appear that biomass accumulation in these cultures depended in the main part on aspartate metabolism since the period during which the fastest uptake of aspartate occurred was also that in which the dry weight increased the most (Figures 3.64 and 3.65). Biomass levels increased very little until 54h, corresponding to the poor use of carbon sources until this time. From 78 to 120h, 50% of the remaining maltose was utilised and α-ketoglutarate levels increased to 156.3mg·L⁻¹ (Figure 3.65). DNA analysis indicated that replicatory growth continued during α-ketoglutarate excretion and beyond 120h. Levels of pyruvate were not great and did not exceed 3.5mg·L⁻¹ recorded at 48-54h. Pyruvate may have leaked from the cells during the catabolism of aspartate which began to disappear rapidly from the medium at this time. Figure 3.65 suggests that α-ketoglutarate was derived principally from maltose, although the initial aspartate utilised may have contributed to α-ketoglutarate before 78h. The results reported in Section 3.3 indicate that carbon from glucose (into which maltose is metabolised) passes at greater levels to pyruvate than α-ketoglutarate. The pH (Figure 3.66) fell in the initial phase of the cultures, rose to 7.0 during rapid amino acid consumption and fell during sugar consumption and acid production to 5.75 (as would be expected; Bader, 1986).

From Figure 3.67 it can be seen that at most, pyruvate represented 0.05% and α-ketoglutarate 3.2% of the consumed carbon sources. Compared to other amino acid containing media, very little of the total consumed carbon was excreted as organic acids. Biomass was in the form of dispersed mycelia with a few pellets throughout these cultures.
Figure 3.64: Graph showing biomass (●) and deoxyribose (▲) changes in a culture grown in SMM containing 15mM maltose and 15mM aspartate.

Figure 3.65: Graph showing changes in carbon and nitrogen sources, maltose (■) and aspartate (▲), and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in SMM containing 15mM maltose and 15mM aspartate.
Figure 3.66: Changes in pH during a culture grown in SMM containing 15mM maltose and 15mM aspartate.

Figure 3.67: Graph showing decrease in total carbon (—□—) and proportion of carbon present in organic acids pyruvate (–●–) and α-ketoglutarate (—▲—) expressed as a % of the total carbon consumed during a culture grown in SMM containing 15mM maltose and 15mM aspartate.
Until around 72h, *S. lividans* did not grow well in glycerol-aspartate medium (Figure 3.68). Between 72 and 96h there was a rapid increase in both biomass (dry weight) and DNA. The highest biomass recorded was 2.1g·L⁻¹ DCW which was a slight improvement on glycerol-nitrate medium. After 96h, biomass and DNA levels were stable. Both carbon sources were utilised most rapidly after 72h (Figure 3.69), corresponding to the rapid increase in biomass levels and suggesting that in this medium, both carbon sources were used to fuel biomass accumulation. Aspartate was exhausted by 96h. After 96h, a large amount of glycerol was consumed (to about 80% in total) indicating that active metabolism continued into the stationary phase as usual; and that glycerol carbon contributed heavily to acid formation which was very rapid in the last 24h of the culture. Pyruvate reached levels of 245.7mg·L⁻¹ and α-ketoglutarate 366.7mg·L⁻¹ by 120h. This profile was unlike anything seen before and may be due to the position of entry of glycerol into the glycolytic pathway. During these cultures the pH fluctuated in a similar way to maltose-aspartate medium (Figure 3.70). The pH fell during initial sugar use, rose during rapid aspartate utilisation and fell again as acids accumulated in the medium. Biomass existed as dispersed mycelia with a few visible pellets. From Figure 3.71 it can be seen that by the end of these cultures about 4.1% of the consumed carbon had been converted to pyruvate and 6.2% to α-ketoglutarate.

No acids were produced in media containing fructose and aspartate. These cultures achieved a moderate biomass yield (2.7g·L⁻¹ at 72h, Figure 3.72). Aspartate was exhausted by 72h (Figure 3.73) while around 50% of the fructose was utilised by 120h (more than from fructose-nitrate medium). The pH rose to 7.0 by 72h (data not shown) and the biomass was in the form of dispersed mycelia and pellets throughout. The poor consumption of fructose by *S. lividans* TK24 suggests that this strain does not grow well on this substrate when supplied as the sole carbon source.
Figure 3.68: Graph showing biomass (●) and deoxyribose (▼) changes in a culture grown in SMM containing 60mM glycerol and 15mM aspartate.

Figure 3.69: Graph showing changes in carbon and nitrogen sources, glycerol (■) and aspartate (▼), and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in SMM containing 60mM glycerol and 15mM aspartate.
Figure 3.70: Changes in pH during a culture grown in SMM containing 60mM glycerol and 15mM aspartate.

Figure 3.71: Graph showing decrease in total carbon (—□—) and proportion of carbon present in organic acids pyruvate (●—) and α-ketoglutarate (▲—) expressed as a % of the total carbon consumed during a culture grown in SMM containing 60mM glycerol and 15mM aspartate.
Figure 3.72: Graph showing biomass changes during a culture in SMM containing 30mM fructose and 15mM aspartate.

Figure 3.73: Graph showing changes in carbon and nitrogen sources, fructose (■) and aspartate ( ▽ ) during a culture in SMM containing 30mM fructose and 15mM aspartate.
3.4.3 Summary

The results described in Section 3.4.2 are summarised in Table 3.12. Summaries of previous results can be found in Tables 3.8 and 3.10.

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>MAXIMUM DRY CELL WEIGHT (g*L⁻¹)</th>
<th>MAXIMUM PYRUVATE (mg*L⁻¹)</th>
<th>MAXIMUM α-KETOGLUTARATE (mg*L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>starch-nitrate</td>
<td>2.8 (96h)</td>
<td>23.8 (54h)</td>
<td>57.0 (72h)</td>
</tr>
<tr>
<td>maltose-nitrate</td>
<td>2.7 (120h)</td>
<td>10.8 (96h)</td>
<td>none</td>
</tr>
<tr>
<td>glycerol-nitrate</td>
<td>2.0 (120h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>fructose-nitrate</td>
<td>1.6 (120h)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>starch-aspartate</td>
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<td>707.1 (78h)</td>
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<td>starch-alanine</td>
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<td>280.0 (54h)</td>
<td>298.0 (78h)</td>
</tr>
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<td>maltose-aspartate</td>
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<td>3.52 (48h)</td>
<td>156.3 (120h)</td>
</tr>
<tr>
<td>glycerol-aspartate</td>
<td>2.1 (96h)</td>
<td>245.7 (120h)</td>
<td>366.7 (120h)</td>
</tr>
<tr>
<td>fructose-aspartate</td>
<td>2.7 (72h)</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

Table 3.12: Summary of results presented in Section 3.4.2. Data shown are maximum recorded values with times in parentheses.
3.5 Shake Flask Cultures Using Complex Media

3.5.1 Objective

The next stage of experimentation was to characterise organic acid production by *S. lividans* TK24 when grown in complex medium. The medium chosen was MEP with 10mM phosphate (PO$_4^{3-}$), used previously for *S. lividans* TK24 by Wrigley-Jones (1991). From these experiments it was possible to identify acids excreted and to establish if they were the same as those excreted in defined cultures, or whether the metabolism of *S. lividans* differed significantly on complex substrates.

Initial tests were done using complete MEP medium with and without 10mM PO$_4^{3-}$ (media 1 and 2 in Table 3.13), because it had previously been found that the growth of *S. lividans* TK24 in this medium varied depending on phosphate levels (Wrigley-Jones, 1991). It was known that acid production was dependent on the presence of a rapidly-used carbohydrate carbon source and was affected by the nitrogen source (Sections 3.2 and 3.4) and that amino acids contributed significant carbon to organic acids (Section 3.3). MEP medium was composed of malt extract broth (MEB), supplying mainly the carbohydrates maltose, glucose, dextrin and sucrose (in decreasing concentration); and bacteriological peptone supplying amino acids and other nitrogen sources on breakdown by the organism. It has been noted that TK24 did not grow on sucrose or dextrin. Glycerol was added as the primary carbon source. Previous experiments (Section 3.4) had characterised acid production in glycerol-based media. In the current set of shake flasks, MEP was broken down into the two other major constituents to investigate acid production (media 3-7 in Table 3.13).

From the manufacturer’s notes it was found that MEB consisted of 85% malt extract (ME) and 15% mycological peptone. It was thought likely that amino acids from the myco-peptone would affect organic acid production so cultures were also grown in media containing ME (media 8 and 9). ME did not sterilise well, forming a cloudy suspension, which may explain why Wrigley-Jones (1991) modified the MEP medium to contain MEB rather than ME as used by Reading & Cole (1977). Finally the effect of an ammonium supplement on acid production by *S. lividans* in complex medium was investigated (medium 10).

It has been found by other researchers (e.g. Adams *et al.*, 1984) that chromatograms of complex medium samples may be too complicated to correctly determine organic acids. Fortunately using the Aminex column (Section 2.1.6) samples...
from MEP, MEB or ME-based media gave simple chromatograms (see Figure 3.1b) from which carbohydrates and organic acids could be identified and measured. Chromatogram simplicity was probably due to the simple mix of sugars in these media and the inability of this column to separate amino acids.

Using the Ultrasphere column, MEP and peptone-based media gave very complex chromatograms due to the high number of amino acids in these media. ME and MEB-based media gave simpler chromatograms. This was very useful since it meant that added amino acids could be correctly identified and measured. No extra amino acids were added to MEP or peptone media. It was not possible to use the Ultrasphere column for back-up measurement of organic acids in these media (1, 2 and 10) but identities were confirmed by enzymic assays. By this stage it had been concluded that the measurement of organic acids using the Aminex column was very accurate.

Each experiment was repeated at least twice. Comparison of all data showed that values varied on average by less than 12.0%. Trends were the same between flasks and at maxima (e.g. highest acid concentrations) data varied by less than 3.0%.

<table>
<thead>
<tr>
<th>No.</th>
<th>MEDIUM COMPOSITION</th>
<th>DESIGNATION</th>
<th>INITIAL C (g·L⁻¹)</th>
<th>INITIAL N (g·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MEP with 10mM PO₄³⁻</td>
<td>MEP(10mM PO₄³⁻)</td>
<td>18.34</td>
<td>2.10</td>
</tr>
<tr>
<td>2</td>
<td>MEP without phosphate supplement</td>
<td>MEP</td>
<td>18.81</td>
<td>2.27</td>
</tr>
<tr>
<td>3</td>
<td>SMM with 10g·L⁻¹ MEB and 30mM nitrate</td>
<td>MEB-nitrate</td>
<td>3.70</td>
<td>0.87</td>
</tr>
<tr>
<td>4</td>
<td>SMM with 10g·L⁻¹ MEB and 15mM glutamate</td>
<td>MEB-glutamate</td>
<td>5.00</td>
<td>0.72</td>
</tr>
<tr>
<td>5</td>
<td>SMM with 10g·L⁻¹ MEB and 15mM aspartate</td>
<td>MEB-aspartate</td>
<td>4.44</td>
<td>0.65</td>
</tr>
<tr>
<td>6</td>
<td>SMM with 10g·L⁻¹ MEB</td>
<td>MEB</td>
<td>3.91</td>
<td>0.47</td>
</tr>
<tr>
<td>7</td>
<td>SMM with 10g·L⁻¹ peptone and 30mM nitrate</td>
<td>peptone-nitrate</td>
<td>7.03</td>
<td>2.11</td>
</tr>
<tr>
<td>8</td>
<td>SMM with 10g·L⁻¹ ME and 30mM nitrate</td>
<td>ME-nitrate</td>
<td>2.91</td>
<td>0.55</td>
</tr>
<tr>
<td>9</td>
<td>SMM with 10g·L⁻¹ MEB and 15mM aspartate</td>
<td>ME-aspartate</td>
<td>3.65</td>
<td>0.35</td>
</tr>
<tr>
<td>10</td>
<td>MEP with 10mM PO₄³⁻ and 15mM ammonium</td>
<td>MEP(10mM PO₄³⁻)-ammonium</td>
<td>18.60</td>
<td>2.55</td>
</tr>
</tbody>
</table>

Table 3.13: Media used in the shake flask cultures described in Section 3.5. Initial carbon and nitrogen was calculated from analysis of samples of uninoculated medium.
3.5.2 Results

3.5.2.1 MEP Medium With and Without Phosphate Supplement

*S. lividans* TK24 grew more readily in complex MEP(10mM PO$_4^{3-}$) medium than in defined media. As shown in Figure 3.74, biomass levels reached around 5.8g·L$^{-1}$ dry weight at 96h, after which levels were stable. Biomass was in the form of dispersed mycelia with very few pellets visible. DNA analysis indicated that replicatory growth continued beyond 120h, possibly supported by glycerol since a fair amount of this carbon source remained in the medium. Figure 3.75 shows the utilisation of those carbon sources which were easily determined by HPLC against earlier standards (glucose, maltose and glycerol). Sucrose and dextrin were not measured. Peptone components were found to be too numerous to determine. Glucose and maltose began to be utilised rapidly after 30h and the period of most rapid uptake matched the period of greatest biomass increase (30-48h). A lag phase of around 24h was seen in virtually all complex or defined shake flask cultures and seems to be specific to the growth of *S. lividans*. In these cultures glucose was exhausted by 48h although the initial concentration was lower than in most defined media at around 4mM. The rapid use of maltose (initially around 9mM) continued until 78h, after which very little more of this component was consumed. In total around 60% of the maltose was utilised. About 85% of the glycerol was consumed by 120h. Very little of this source was taken up until after glucose was exhausted, indicating preferential or sequential use of mixed carbon sources by *S. lividans* (glucose and maltose, then glycerol after glucose exhaustion with little further use of maltose; Figure 3.75).

During these cultures pyruvate and α-ketoglutarate were produced (Figure 3.76). No acids other than these were detected in complex medium, showing that *S. lividans* TK24 produces exclusively these acids in the media used in this project. A maximum pyruvate level of 155.9mg·L$^{-1}$ was recorded at 48h and maximum α-ketoglutarate of 1013.9mg·L$^{-1}$ at 72h. High α-ketoglutarate production had been expected based on previous observations, due to the contribution of carbon from amino acids in the peptones (by weight, amino acids make up 70-75% of the peptones). During acid production the pH fell to 4.5 (Figure 3.77). A sharp fall in pH from 6.5 to 5.5 accompanied pyruvate accumulation, with a further fall from 5.5 to 4.5 accompanying α-ketoglutarate production. The pH rose to 5.0 by 120h with the reuse of some of the α-ketoglutarate. The pyruvate was exhausted by 96h, again indicating a preference by the
Figure 3.74: Graph showing biomass (—•—) and deoxyribose (—▼—) changes during a culture grown in MEP medium with a 10mM PO₄³⁻ supplement.

Figure 3.75: Graph showing changes in selected carbon sources, glycerol (—▽—), glucose (—□—) and maltose (—●—) during a culture grown in MEP medium with a 10mM PO₄³⁻ supplement.
Figure 3.76: Graph showing changes in organic acids, pyruvate (●) and α-ketoglutarate (▲) during a culture grown in MEP medium with a 10mM PO$_4^{3-}$ supplement.

Figure 3.77: Changes in pH during a culture grown in MEP medium with a 10mM PO$_4^{3-}$ supplement.
organism for pyruvate as a carbon source. However, some authors have suggested that pyruvate may be required as an aid for ammonium assimilation at low concentrations via alanine dehydrogenase (Shapiro & Vining, 1983).

Less of each acid was excreted than may have been expected from the rich nature of the medium. Compared to defined media, not all of the supplied carbon was consumed. The complexity of the medium prevented the determination from HPLC of exactly how much carbon remained unused and an unfortunate equipment malfunction prevented carbon analysis (Section 2.1.10) at the time of culturing. Later shake flask cultures in this medium (Section 3.6) were conducted with parallel carbon analysis. These indicated that around 40% of the initial carbon was consumed and that of this up to 1.2% passed to pyruvate and 4.9% to α-ketoglutarate.

Both glycerol and α-ketoglutarate were taken up rapidly from the medium until 120h although biomass did not increase much in this period. Heavy foaming from 72h onwards indicated lysis of material. Also, although the pH fell more drastically in these cultures (Figure 3.77), the continued use of glycerol and α-ketoglutarate until 120h indicated that the culture was not killed by the low pH. The pH effects may have been buffered by the extra phosphate, as suggested by Wrigley-Jones (1991).

When cultured in MEP medium without phosphate supplement, S. lividans did not grow as well. This was also reported by Wrigley-Jones (1991) who found that acid production was the same in supplemented and unsupplemented medium, and who speculated that the phosphate acted as a buffer enabling MEP(10mM P04^3-) cultures to achieve higher biomass levels. In MEP cultures without an extra phosphate supplement the biomass reached 3.5g·L^-1 dry weight by 120h (Figure 3.78) and was in the form of dispersed mycelia with few pellets. Growth was slower and may reflect the reduced utilisation of maltose and glycerol from these cultures, although glucose was exhausted by 48h as before (Figure 3.79). Less than half of the initial maltose or glycerol was consumed. The acid production profile also differed significantly (Figure 3.80). More pyruvate but less α-ketoglutarate was produced, at maxima of 233.4mg·L^-1 (54h) and 480.7mg·L^-1 (120h) respectively. The pH fell to 4.5 (Figure 3.81) and remained so for longer than in MEP(10mM P04^3-) medium which may, combined with low carbon source consumption, have prevented greater biomass accumulation. Although less acid was excreted, the pH fall was similar to the phosphate supplemented cultures, probably
Figure 3.78: Graph showing biomass (■) and deoxyribose (▼) changes during a culture grown in MEP medium without a PO₄³⁻ supplement.

Figure 3.79: Graph showing changes in selected carbon sources, glycerol (▼), glucose (□) and maltose (○) during a culture grown in MEP medium without a PO₄³⁻ supplement.
Figure 3.80: Graph showing changes in organic acids, pyruvate (—●—) and α-ketoglutarate (—■—) during a culture grown in MEP medium without a PO₄³⁻ supplement.

Figure 3.81: Changes in pH during a culture grown in MEP medium without a PO₄³⁻ supplement.
due to the absence of phosphate buffering effects. Also, extrapolation of the plot in Figure 3.80 suggests that pyruvate may have reached around 400mg L\(^{-1}\).

Not all of the carbon sources were exhausted. It is not known exactly how much was consumed due to equipment malfunction, but general indications were that less consumed substrate carbon was excreted as organic acids by cultures grown in MEP complex medium compared to defined media.

In both types of MEP medium described in this section and in all complex cultures described in Sections 3.5 and 3.6, organic acid excretion began at the point when the glucose component of the ME began to be taken up rapidly from the medium at around 30h. In all complex cultures, glucose was exhausted by 48 to 54h, while the utilisation profile of other carbon sources varied between media (compare Figures 3.75 and 3.79 for example). It can reasonably be concluded that in ME-based media acidification was initiated by the metabolism of glucose. Acid levels were subsequently increased or affected by the metabolism of other carbon sources derived from ME and peptone. Some of these sources may have ‘interactive’ metabolic effects as mentioned in Section 3.2 and 3.4. These results will be discussed further in Section 4.
3.5.2.2 Breakdown of MEP Medium to MEB and Peptone

The complex constituents of MEP medium, MEB and bacto-peptone, were tested individually. Glycerol had been tested previously (Section 3.4). MEB and peptone were initially combined with nitrate as an extra nitrogen source and made up with an SMM base to supply any minerals and trace elements lacking. Whereas MEP(10mM PO₄³⁻) medium contained a supplement of 1.74g-L⁻¹ K₂HPO₄ supplying around 10mM phosphate compounds, SMM contained 1.5g-L⁻¹ K₂HPO₄ and 3.0g-L⁻¹ NaH₂PO₄, supplying around 34mM phosphate compounds. Therefore media containing complex constituents in an SMM base were sufficiently phosphate buffered and could be compared directly with MEP(10mM PO₄³⁻) cultures.

In MEB-nitrate medium, *S. lividans* TK24 grew moderately well and achieved a biomass yield of 2.3g-L⁻¹ dry weight at 48h (Figure 3.82) which was comparable to defined media but considerably less than MEP(10mM PO₄³⁻) medium. This may be partly due to the use of nitrate which was less readily utilised by *S. lividans* than amino acids (or peptone). The initial available carbon and nitrogen was also less. Maltose from the MEB was poorly used with around 30% consumed by 120h (data not shown). Glucose was exhausted by 48h. The amino acids in the 15% myco-peptone portion of MEB were too numerous to determine, while nitrate was present throughout.

A large amount of organic acid was excreted which was interesting considering the poor use of the major carbon sources. The acid production profile was unlike any seen before (Figure 3.83) although the proportions of acids were similar to glucose-nitrate medium with maximum pyruvate levels greatly exceeding α-ketoglutarate levels. Up to 121.3mg-L⁻¹ α-ketoglutarate was produced by 48h, and this was virtually reused by 54h. The unusual rapidity of this reuse may be related to the poor use of the other carbon sources or the low levels excreted. In contrast, pyruvate levels reached 591.8mg-L⁻¹ between 30 and 48h and then continued to increase gradually to a maximum of 771.4mg-L⁻¹ at 96h. Since apparently very little of the available carbon sources were consumed then these concentrations represent a large proportion of that carbon actually metabolised, which may again reflect the low biomass yield. Biomass existed as a mixture of pellets and dispersed mycelia. The pyruvate profile suggested that very little pyruvate was reassimilated during stationary phase (which began at around 48h according to DNA analysis; Figure 3.82). The pH fell to 5.0 (Figure 3.84) and stayed low for the remainder of the cultures. It could be suggested that the low pH
Figure 3.82: Graph showing biomass (—•—) and deoxyribose (— —) changes during a culture grown in SMM containing 10g-L⁻¹ MEB and 30mM nitrate.

Figure 3.83: Graph showing changes in organic acids, pyruvate (—•—) and α-ketoglutarate (— ▲—) during a culture grown in SMM containing 10g-L⁻¹ MEB and 30mM nitrate.
Figure 3.84: Changes in pH during a culture grown in SMM containing 10g·L⁻¹ MEB and 30mM nitrate.

Figure 3.85: Graph showing biomass (—•—) and deoxyribose (—▼—) changes during a culture grown in SMM containing 10g·L⁻¹ peptone and 30mM nitrate.
killed the culture, although the pH fell lower in MEP(10mM \(\text{PO}_4^{3-}\)) medium (Section 3.5.2.1) and an equivalently low pH did not affect later cultures (Sections 3.5.2.2 and 3.5.2.3).

Peptone-nitrate medium did not support acid excretion. The biomass yield improved slightly from MEB-nitrate medium, probably due to the extra initial carbon and nitrogen available. The biomass reached 2.7g·L\(^{-1}\) at 54h (Figure 3.85). These results indicate firstly the importance of peptone as a substrate for biomass accumulation in MEP medium; and secondly that a combination of complex medium components is required to achieve the biomass levels observed in MEP(10mM \(\text{PO}_4^{3-}\)) medium. The pH rose steadily throughout these cultures to pH 8.0 (data not shown) as would be expected from the metabolism of nitrate and amino acids by microbes (Bader, 1986). Nitrate was in excess throughout these cultures. The biomass was in the form of dispersed mycelia and pellets throughout. Due to the lack of acid production on peptone, no alternatives (e.g. tryptone, casamino acids) were tested.

Subsequent cultures were conducted using MEB combined with an extra amino acid to partially mimic the effect of added peptone. It was suspected that the acid profiles would change and probably increase and that biomass yields would improve, since amino acids appeared to be more readily used than nitrate as nitrogen sources. Analysis of the peptones present in MEP showed that both contained a greater amount of glutamate than any other amino acid (12.1\% and 8.8\% in myco- and bacto-peptone respectively out of a total amino acid weight content of 71.2 and 74.3\% respectively; Oxoid Manual, 5th edition, 1982). Hence, the first cultures were grown in MEB-glutamate medium. Of the amino acids tested previously, alanine and aspartate were present at similar levels (alanine 5.2 and 6.1\% and aspartate 6.8 and 8.3\% in myco- and bacto-peptone respectively), therefore only aspartate was chosen for further testing in MEB-aspartate medium.

The replacement of nitrate with glutamate gave a higher biomass yield to 5.2g·L\(^{-1}\) by 72h (Figure 3.86). DNA analysis indicated that replicatory growth also ended at 72h. Biomass was in the form of dispersed mycelia and a few pellets throughout. Heavy foaming occurred after 72h, possibly associated with the 40\% drop in biomass between 72 and 120h. Unlike MEB-nitrate medium, the readily-measured carbon sources were exhausted rapidly (Figure 3.87). Glucose was exhausted by 48h and maltose effectively
Figure 3.86: Graph showing biomass (––) and deoxyribose (–––) changes during a culture grown in SMM containing 10g·L⁻¹ MEB and 15mM glutamate.

Figure 3.87: Graph showing changes in selected carbon sources, glucose (–○–) and maltose (–■–) and glutamate (–▲–) during a culture grown in SMM containing 10g·L⁻¹ MEB and 15mM glutamate.
Figure 3.88: Graph showing changes in organic acids, pyruvate (—■—) and α-ketoglutarate (▲) during a culture grown in SMM containing 10g·L⁻¹ MEB and 15mM glutamate.

Figure 3.89: Changes in pH during a culture grown in SMM containing 10g·L⁻¹ MEB and 15mM glutamate.
by 72h. Since glutamate was also exhausted by 54h, this may explain the cessation of rapid growth at 72h. Again, the results point to some sort of metabolic effect of carbon and nitrogen sources since *S. lividans* utilised the available carbon sources much better in MEB-glutamate medium than MEB-nitrate medium. The poor utilisation of maltose from MEP medium may have been influenced by the presence of excess glycerol; while poor carbon use in MEB-nitrate medium may have been related to the presence of nitrate when both are compared to the rapid uptake of maltose from MEB-amino acid media.

Pyruvate was excreted, reached a maximum of 202.5mg·L⁻¹ at 54h and was exhausted by 78h (Figure 3.88). In these cultures pyruvate appeared to be metabolised as a carbon source to support rapid growth as in some defined media. α-Ketoglutarate was also excreted, at maximal rates during the most rapid carbon sources uptake, and peaked at 524.5mg·L⁻¹ at 72h. This acid was also reused rapidly and almost to completion by 120h, possibly to support active metabolism in the stationary phase. The pH did not fall very far (Figure 3.89) although acid levels were equivalent to those in full MEP medium. This was possibly due to a buffering effect of the extra phosphate from SMM. When α-ketoglutarate was rapidly reused, the pH rose to reach over 7.0 by the end of the culture.

The acid profile in MEB-glutamate medium was effectively the same as for other carbon source-amino acid media although it is suspected that excreted acids accounted for less of the consumed carbon since the initial availability was greater. Similar profiles were obtained for MEB-aspartate medium. In these cultures, biomass levels reached up to 4.6g·L⁻¹ dry weight by 96h (Figure 3.90). Material was a mixture of dispersed mycelia and pellets. As with MEB-glutamate medium, the end of biomass accumulation was preceded by the exhaustion of glucose, aspartate and the majority of the maltose from the medium (Figure 3.91) although other carbon sources were not determined. Each event occurred around 24 hours later than in MEB-glutamate cultures. Acid peaks were also recorded later (Figure 3.92), pyruvate at 72h (135.6mg·L⁻¹) and α-ketoglutarate at 96h (337.5mg·L⁻¹). Pyruvate was again completely reassimilated before the end of rapid growth. α-Ketoglutarate peaked at the time of the biomass peak and around 50% was reused in the following 24h. Extrapolation of the lines in Figure 3.92 suggests that pyruvate may have reached levels over 200mg·L⁻¹ and α-ketoglutarate levels of over 500mg·L⁻¹. This would be in keeping with the greater acid concentrations produced by
Figure 3.90: Graph showing biomass (●) and deoxyribose (▼) changes during a culture grown in SMM containing 10g·L⁻¹ MEB and 15mM aspartate.

Figure 3.91: Graph showing changes in selected carbon sources, glucose (○) and maltose (○) and aspartate (▼) during a culture grown in SMM containing 10g·L⁻¹ MEB and 15mM aspartate.
Figure 3.92: Graph showing changes in organic acids, pyruvate (–○–) and α-ketoglutarate (–▲–) during a culture grown in SMM containing 10g·L⁻¹ MEB and 15mM aspartate.

Figure 3.93: Changes in pH during a culture grown in SMM containing 10g·L⁻¹ MEB and 15mM glutamate.
glucose-aspartate cultures compared to glucose-glutamate cultures; and the fact that the pH fell lower in MEB-aspartate cultures (Figure 3.93) than MEB-glutamate cultures (Figure 3.89).

Although it is unfortunately not known exactly how much of the initial carbon was utilised from MEB-based media, it is obvious that the carbon excreted in the acids represented a greater proportion of that consumed compared to MEP media, since the initial levels were less (see Table 3.13).

A final set of cultures were grown in SMM containing only MEB with no extra nitrogen source. It was calculated that MEB itself supplied 0.42g.L\(^{-1}\) nitrogen so cultures may have been nitrogen-limited. As can be seen from Figure 3.94, \textit{S. lividans} achieved higher biomass levels in this medium than in MEB-nitrate medium. This indicated that \textit{S. lividans} grows very poorly on and may even be inhibited by nitrate. Biomass levels increased slowly but reached 3.0g.L\(^{-1}\) by 120h. Biomass material was in the form of an equal mixture of pellets and dispersed mycelia. Glucose was exhausted rapidly, while maltose was consumed more rapidly than MEB-nitrate medium with approximately 80% used by 120h. Little maltose was used until the glucose was exhausted (Figure 3.95).

Both pyruvate and \(\alpha\)-ketoglutarate were excreted by these cultures, although at low levels compared to other complex cultures (Figure 3.96). The levels of \(\alpha\)-ketoglutarate were higher as expected in the absence of nitrate but with the contribution of carbon from the myco-peptone. At the simplest level the pyruvate and \(\alpha\)-ketoglutarate profiles resembled those from cultures in MEB-amino acid media. Pyruvate reached a peak of 116.2mg.L\(^{-1}\) at 54h and was reused until exhaustion at 120h. By continuing the cultures until 144h, it was known that the biomass peak was at 120h, which again indicated that pyruvate was reassimilated by the organism to fuel biomass accumulation. \(\alpha\)-Ketoglutarate again reached maximum concentrations at the same time as the biomass peak (277.6mg.L\(^{-1}\) at 120h). Levels were high and stable from 72h onwards, suggesting poor \(\alpha\)-ketoglutarate reuse during the period of greatest maltose uptake from 72 to 120h (Figure 3.96). The pH fell to 5.0 and remained low due to the high \(\alpha\)-ketoglutarate concentrations in the medium. (Figure 3.97). A slight rise in pH between 78 and 96h reflected the reuse of pyruvate although \(\alpha\)-ketoglutarate levels continued to rise.
Figure 3.94: Graph showing biomass (---) and deoxyribose (--) changes during a culture grown in SMM containing 10g·L⁻¹ MEB only.

Figure 3.95: Graph showing changes in selected carbon sources, glucose (---) and maltose (--) during a culture grown in SMM containing 10g·L⁻¹ MEB only.
Figure 3.96: Graph showing changes in organic acids, pyruvate (—at-) and \(\alpha\)-ketoglutarate (—k—) during a culture grown in SMM containing 10g-L\(^{-1}\) MEB only.

Figure 3.97: Changes in pH during a culture grown in SMM containing 10g-L\(^{-1}\) MEB only.
These results show that at the simplest level, trends of acid production in complex media resembled those in defined media containing a readily used carbon source and either nitrate or amino acids as the sole nitrogen source. It was suspected that the proportion of consumed carbon passing to acids was equivalent to or slightly less (but no more) than that in defined media. These results will be discussed in Section 4.
3.5.2.3 Breakdown of MEP Medium to ME

MEB was found to contain 85% malt extract and 15% peptone, containing amino acids which could affect organic acid production by S. lividans. The organism was also grown in media containing pure ME combined with defined inorganic and organic nitrogen sources.

Although ME formed a cloudy medium on autoclaving, the organism achieved greater biomass levels in ME-nitrate medium than MEB-nitrate medium (Figure 3.98), perhaps due to the increased availability of carbohydrates which make up the major part of ME. The biomass yield was relatively low for a complex medium (3.5g·L⁻¹ dry weight at 78h) which was probably due to the use of nitrate. Around 90% of the maltose was consumed between 48 and 96h, with the most rapid increases in biomass and acid concentrations occurring in this period (Figure 3.99). DNA analysis (Figure 3.98) showed replicatory growth to continue until 96h, corresponding to the rapid utilisation of maltose until this time. Glucose was exhausted by 48h. Both the rapid utilisation of glucose and the excretion of pyruvate and α-ketoglutarate began at around 30h.

The acid production profile (Figure 3.100) was similar to both glucose-nitrate and MEB-nitrate media with more pyruvate excreted than α-ketoglutarate. Levels were lower than the earlier media even though the initial amounts of glucose and maltose were greater and more maltose was utilised from ME-nitrate medium. Pyruvate peaked at 366.3mg·L⁻¹ at 72h and α-ketoglutarate at 87.7mg·L⁻¹ at 48h. It had been expected that acid production would be greater in this medium due to the greater glucose availability, and to see a trend whereby acid production was greatest in glucose-nitrate cultures, less in ME-nitrate cultures and lowest in MEB-nitrate cultures. Since acid levels were highest in the latter medium, this suggests either the contribution of carbon from the amino acids in the peptone part of MEB to organic acids; or that conversion of available carbohydrate carbon was less efficient in this medium. The maximum biomass achieved in MEB-nitrate medium was poor (2.3g·L⁻¹ dry weight) considering that it supplied the highest initial carbon and nitrogen of the three types. Lower than expected excretion of acids may indicate that conversion of carbon to biomass was more efficient in ME-nitrate medium, since the maximum biomass was higher than glucose-nitrate or MEB-nitrate media.

Pyruvate was rapidly reassimilated and completely gone by 96h, suggesting its use as a carbon source supporting biomass accumulation. α-Ketoglutarate was reused.
Figure 3.98: Graph showing biomass (—•—) and deoxyribose (—▼—) changes during a culture grown in SMM containing 10g·L\(^{-1}\) ME and 30mM nitrate.

Figure 3.99: Graph showing changes in selected carbon sources, glucose (—○—) and maltose (—□—) during a culture grown in SMM containing 10g·L\(^{-1}\) ME and 30mM nitrate. Nitrate not shown but present throughout.
Figure 3.100: Graph showing changes in organic acids, pyruvate (- - -) and α-ketoglutarate (Δ - Δ) during a culture grown in SMM containing 10g·L⁻¹ ME and 30mM nitrate.

Figure 3.101: Changes in pH during a culture grown in SMM containing 10g·L⁻¹ ME and 30mM nitrate.
steadily to exhaustion by 120h although this most likely reflects the low levels excreted rather than faster reassimilation. Again it was not possible to measure the amount of carbon consumed but since virtually all of the readily-measurable carbon was consumed it can be assumed that a greater amount of the consumed carbon passed to acids than in MEP medium, but less than MEB-nitrate medium (see above) or glucose-nitrate medium. The pH fell to 5.5 at the highest pyruvate levels and rose to 7.5 by the end of the culture (Figure 3.101). The pH increased during α-ketoglutarate formation, although a greater increase occurred during the reuse of this metabolite from 72 to 120h. Nitrate was in excess throughout but metabolism of this source may also explain the pH increase. The biomass was observed to be a mixture of dispersed mycelia and pellets.

*S. lividans* grew well in ME-aspartate medium, although not as well as in MEB-amino acid media which would be expected due to the lower initial carbon. Cultures achieved biomass levels of around 4.0g·L⁻¹ dry weight by 72h (Figure 3.102) and DNA analysis showed that replicatory growth ended around 72 to 78h. Biomass existed as dispersed mycelia and pellets. From Figure 3.103 it can be seen that, as with MEB-aspartate media, all of the major carbon sources were consumed within 120h. Since this also occurred in ME-nitrate medium, the very poor use of maltose in all repeats of MEB-nitrate cultures remains an obscure anomaly. Also maltose was consumed more slowly in ME-aspartate cultures than MEB-aspartate cultures (compare Figures 3.103 and 3.91) which cannot easily be explained since maltose was utilised faster from ME-nitrate than MEB-nitrate media.

Acid concentrations were elevated in ME-aspartate cultures compared to the equivalent MEB cultures. The acid production trend which had been expected in nitrate-based media (glucose-nitrate greater than ME-nitrate greater than MEB-nitrate) was instead observed in aspartate-based media, which again pointed to the MEB-nitrate cultures being an odd anomaly. Acid production was generally greatest in glucose-amino acid media (Section 3.2), lower in MEB-amino acid cultures (Section 3.5.2.2) and intermediate in ME-amino acid cultures. In ME-aspartate media, pyruvate reached a concentration of 297.6mg·L⁻¹ and α-ketoglutarate 574.2mg·L⁻¹ respectively, both at 78h (Figure 3.104). The peak in extracellular pyruvate concentration was later and higher than previous cultures and this acid was not exhausted before the biomass maximum. The pH fell to 5.5 (Figure 3.105) corresponding to maximum pyruvate and α-ketoglutarate levels, and rose as pyruvate was reassimilated.
Figure 3.102: Graph showing biomass (---) and deoxyribose (—) changes during a culture grown in SMM containing 10g·L⁻¹ ME and 15mM aspartate.

Figure 3.103: Graph showing changes in selected carbon sources, glucose (□□) and maltose (○○) and aspartate (—) during a culture grown in SMM containing 10g·L⁻¹ ME and 15mM aspartate.
Figure 3.104: Graph showing changes in organic acids, pyruvate (---) and \( \alpha \)-ketoglutarate (\( \Delta \)) during a culture grown in SMM containing 10g·L\(^{-1}\) ME and 15mM aspartate.

Figure 3.105: Changes in pH during a culture grown in SMM containing 10g·L\(^{-1}\) ME and 15mM aspartate.
An observation which is consistent between all of the media described in Sections 3.2 to 3.5 was that S. lividans TK24 excreted exclusively pyruvic and α-ketoglutaric acids as metabolic by-products. Apparent indications were that a greater proportion of the consumed carbon passed to acids as the medium became increasingly complex. This was contrary to the initial expectation that acid production would increase with increasing carbon source availability. In defined media containing glucose with any nitrogen source, there was a lower initial carbon supply, of which all was consumed in most cases. Acid production was generally very high and thus a larger proportion of consumed carbon passed to acids than in MEP medium. The initial proportion of consumed carbon passed to acids in MEP medium was low. Hence, a greater proportion of consumed carbon was not apparent and acid production was quite low. In contrast, in MEP media containing MEB with any nitrogen source, the initial carbon content was the highest and acid production accounted for a very small proportion of it (around 6%); Section 3.6). In media containing MEB and any nitrogen source but excluding the anomalous MEB-nitrate cultures), the initial carbon content was higher than defined media, more of this was consumed and initial proportion of consumed carbon passed to acids in MEP medium was greater than defined media. It can be assumed that a greater proportion of consumed carbon passed to acids than MEP or MEB media but again less than defined media.
3.5.2.4 Effect of Ammonium Ions in Phosphate Supplemented MEP Medium

Since ammonium ions were found to prevent acid production by *S. lividans* in defined media, the organism was grown in MEP(10mM PO$_4^{3-}$) medium containing a 15mM ammonium supplement to see if ammonium would also reduce or prevent acid over production in complex media.

There were two main differences. Firstly the biomass yield increased to reach a peak of 6.0g.L$^{-1}$ at 96h (Figure 3.106). Secondly, acid production was reduced consistently by a total of over 80%. The maximum pyruvate recorded was 24.7mg.L$^{-1}$ at 48h and the maximum $\alpha$-ketoglutarate 172.4mg.L$^{-1}$ at 54h (Figure 3.107). The pyruvate maximum was reduced by 84% compared to non-ammonium-supplemented medium and the $\alpha$-ketoglutarate maximum by 83%. At the maxima acids accounted for no more than 1.1% of the consumed carbon sources (based on that described in Section 3.6). The pH fell only to 5.5-6.0 (Figure 3.108) and was generally more stable than in previous MEP cultures.

The utilisation of the readily-measured carbon sources (data not shown) was essentially identical to that from previous MEP(10mM PO$_4^{3-}$) cultures (Figure 3.75). Glucose was exhausted by 48h; glycerol levels were unchanged until 54h and then decreased rapidly until around 75% was used by 120h; and around 50% of the maltose was used principally in the period 24 to 96h. The ammonium supply was in excess throughout, although around 70% was used, mainly in the first 54h.

Overall, the results demonstrate that acid production by *S. lividans* is supported by nitrogen sources including nitrate, amino acids and peptides from complex substrates. The presence of a large amount of ammonium ions prevents or reduces acid excretion. Analysis of myco-peptone and bacto-peptone show that in MEP(10mM PO$_4^{3-}$) medium these components supply around 0.09g.L$^{-1}$ ammonia. The effect of this, and of any transaminated ammonium generated from the degradation of amino acids, appears to be insignificant to that caused by a 2g.L$^{-1}$ ammonium salt supplement, supplying 0.55g.L$^{-1}$ ammonium ions. This will be discussed further in Section 4.
Figure 3.106: Graph showing biomass (—) and deoxyribose (—) changes during a culture grown in MEP medium with 10mM $PO_4^{3-}$ and 15mM $(NH_4)_2SO_4$ supplements.

Figure 3.107: Graph showing changes in organic acids, pyruvate (—) and $\alpha$-ketoglutarate (—) during a culture grown in MEP medium with 10mM $PO_4^{3-}$ and 15mM $(NH_4)_2SO_4$ supplements.
Figure 3.108: Changes in pH during a culture grown in MEP medium with 10mM $PO_4^{3-}$ and 15mM $(NH_4)_2SO_4$ supplements.
3.5.3 Summary

The observations described in Section 3.5.2 are summarised in Table 3.14. Previous results are summarised in Tables 3.8, 3.10 and 3.12.

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>MAXIMUM DRY CELL WEIGHT (g·L⁻¹)</th>
<th>MAXIMUM PYRUVATE (mg·L⁻¹)</th>
<th>MAXIMUM α-KETOGLUTARATE (mg·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP(10mM PO₄³⁻)</td>
<td>5.8 (96)</td>
<td>155.9 (48)</td>
<td>1013.9 (72)</td>
</tr>
<tr>
<td>MEP</td>
<td>3.5 (120)</td>
<td>233.4 (54)</td>
<td>480.7 (120)</td>
</tr>
<tr>
<td>MEB-nitrate</td>
<td>2.3 (48)</td>
<td>771.4 (96)</td>
<td>121.3 (48)</td>
</tr>
<tr>
<td>MEB-glutamate</td>
<td>5.2 (72)</td>
<td>202.5 (54)</td>
<td>524.5 (72)</td>
</tr>
<tr>
<td>MEB-aspartate</td>
<td>4.6 (96)</td>
<td>135.6 (72)</td>
<td>337.5 (96)</td>
</tr>
<tr>
<td>MEB</td>
<td>3.0 (120)</td>
<td>116.2 (54)</td>
<td>277.6 (120)</td>
</tr>
<tr>
<td>peptone-nitrate</td>
<td>2.7 (54)</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>ME-nitrate</td>
<td>3.5 (78)</td>
<td>366.3 (72)</td>
<td>87.7 (48)</td>
</tr>
<tr>
<td>ME-aspartate</td>
<td>4.0 (72)</td>
<td>297.6 (78)</td>
<td>574.2 (78)</td>
</tr>
<tr>
<td>MEP(10mM PO₄³⁻)-ammonium</td>
<td>6.0 (96)</td>
<td>24.7 (48)</td>
<td>172.4 (54)</td>
</tr>
</tbody>
</table>

Table 3.14: Summary of results presented in Section 3.5.2. Data shown are maximum recorded values with times in parentheses.
3.6 Total Carbon and Nitrogen Balancing

3.6.1 Objective

A pair of shake flask cultures in MEP(10mM PO₄²⁻) medium were used to gather carbon and nitrogen data. This was used for the construction of maps showing the proportion of carbon passing along the different metabolic routes to organic acids, biomass/storage materials and CO₂ during growth in this medium which was used by Wrigley-Jones (1991).

The following data was obtained using repeat samples: biomass (DCW) in g·L⁻¹; organic acids in mg·L⁻¹ and mg·L⁻¹ of component carbon calculated from HPLC; total carbon and nitrogen in whole broth (C_w/N_w); total carbon and nitrogen in the extracellular medium (C_m/N_m); and total carbon and nitrogen in biomass (C_b/N_b). In each case the relationship was C_m+C_b=C_w and N_m+N_b=N_w.

The nitrogen content of the cultures was expected to remain constant since none should be lost from them. Thus N_w should be constant, while N_b should increase and N_m should fall during biomass accumulation. Lysis of the mycelia would cause some nitrogen compounds to leak back into the extracellular medium.

From the carbon data the following could be calculated at a selection of sampling points (0, 48, 72, 96 and 120h):

1. The total carbon in the biomass including storage materials (C_b).
2. The total carbon in the medium (C_m) from which was subtracted the amount of carbon present in excreted organic acids (calculated from HPLC analysis).
3. The total amount of carbon lost through respiration to CO₂, calculated from the difference in C_w and C_m at each timepoint against the initial C_w and C_m.

Until 72h these calculations were accurate; but after 72h, foaming indicated that lysis was occurring. Prosser & Tough (1991) note that biomass increase and lysis can occur simultaneously, indicated in the cultures presented in Section 3.6.2 by the continued increase in biomass until 96h. Lysis caused the release of carbon compounds into the medium, hence the C_m values would include this. Although C_m continued to fall, it is possible that carbon levels fell more than indicated (see Section 3.6.2) and that more carbon was lost to CO₂.

Shake flask cultures were used due to the failure of fermentation cultures (Section 3.8). Had the use of fermenters been possible, then formation of CO₂ could have been monitored using off-gas analysis, and loss of consumed carbon as CO₂ could...
have been calculated. In addition, shake flask cultures cannot be compared exactly to
growth in the controlled conditions of a fermenter (see comparison of vessels in Section
4.2.1). Hence, data for carbon in acids and biomass in the shake flasks are accurate and
useful as such; while the CO₂ calculations give at best a qualitative picture. Values are
roughly though not directly comparable to theoretical stirred vessel fermentations.
3.6.2 Results

The fermentation data obtained from the pair of MEP(10mM \( \text{PO}_4^{3-} \)) cultures is presented in Figures 3.109 to 3.112. Data points varied by, on average, less than 12.2\% between the pair with the smallest variation (less than 2.7\%) at the maxima of each parameter (acids, biomass). Variation from the original MEP(10mM \( \text{PO}_4^{3-} \)) cultures (see below) was thought to be due to the use of a fresh batch of MEB and bacto-peptone in these media. Biomass and organic acid values varied by an average of less than 21.7\% from the earlier repeats.

The biomass profile (Figure 3.109) followed the same trends as previous MEP(10mM \( \text{PO}_4^{3-} \)) cultures. The maximum biomass yields recorded were 4.9 and 5.2\text{g.\text{-}L}^{-1} DCW at 96h. DNA analysis again indicated that replicatory growth continued at a slow rate beyond 120h. Biomass was mainly in the form of dispersed mycelia with a few visible pellets. The utilisation of the readily-measured carbon sources mirrored earlier repeats (Figure 3.110). Variations reflected the minor differences in the biomass profiles; for example, maltose was consumed more rapidly from flask 1 in the period 48 to 72h, coincident with the greater increase in DCW in this culture in the same period. The acid production profiles (Figure 3.111) were similar to earlier repeats with two main differences. The maximum concentration of pyruvate was higher and the maximum concentration of \( \alpha \)-ketoglutarate was lower. Pyruvate reached 192.0 and 212.2\text{mg.\text{-}L}^{-1} in flasks 1 and 2 respectively; while \( \alpha \)-ketoglutarate levels reached 904.3 and 774.3\text{mg.\text{-}L}^{-1} respectively. Such variations would be consistent with minor variations in the composition of the MEB and peptone batches, based on earlier observations. Also, acid maxima occurred around 6 hours later than previous repeats (pyruvate at 54h and \( \alpha \)-ketoglutarate at 78h). As a result, the lowest pH values occurred later (78h in flask 1 and 96h in flask 2), although the pH reached 4.5 in both (Figure 3.112). A slight rise in pH occurred as some of the organic acids were reused.

Carbon analysis showed that an average of approximately 7.1\text{g.\text{-}L}^{-1} total carbon was consumed. Excreted pyruvate represented at most 1.2\% and \( \alpha \)-ketoglutarate 4.9\% of the consumed carbon, which is lower than for most of the defined media. The average carbon and nitrogen data obtained from the cultures is given in Tables 3.15 and 3.16. Data varied on average by less than 9.8\% between all samples. The carbon and nitrogen data is plotted in Figure 3.113 and 3.114. The carbon flow through the metabolic pathways was calculated as far as possible at each timepoint and is presented in Figure 3.115. Approximately 39\% of the initial carbon was consumed. Most of the carbon
supply remained unused including, for example, about 38% of the initial glycerol and 43% of the initial maltose. It can be seen from Figures 3.115a-d that at each timepoint, the majority of the consumed carbon was present in the biomass or had been respired to CO₂. The biomass contained an average of 53% carbon and 20% nitrogen which was comparable to defined media. Although the carbon flow could not be exactly balanced, it can be assumed that around 65% of the carbon consumed during the 120h cultures was lost to CO₂, which is also comparable to the defined cultures reported in Section 3.2. Much less of the consumed carbon was passed to excreted organic acids. A maximum of 3.2% of the carbon consumed by the cultures by 48h existed as excreted pyruvate; while a maximum of 4.5% of the carbon consumed by 96h existed as excreted α-ketoglutarate. Since the amount of carbon in both the biomass and acids fell during the whole cultures, and the amount lost to CO₂ rose, it can be assumed that carbon from reassimilated acids was also respired to CO₂.

<table>
<thead>
<tr>
<th>TIME (h)</th>
<th>C_w (g·L⁻¹)</th>
<th>C_m (g·L⁻¹)</th>
<th>C_b (g·L⁻¹)</th>
<th>C_m+C_b (g·L⁻¹)</th>
<th>DIFFERENCE OF C_m+C_b FROM C_w (mg·L⁻¹)</th>
<th>PYRUVATE CARBON (mg·L⁻¹)</th>
<th>KETOGLUT CARBON (mg·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18.67</td>
<td>18.34</td>
<td>0.33</td>
<td>18.67</td>
<td>+0.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>17.68</td>
<td>16.41</td>
<td>1.28</td>
<td>17.69</td>
<td>+0.01</td>
<td>63.4</td>
<td>45.6</td>
</tr>
<tr>
<td>54</td>
<td>16.85</td>
<td>15.23</td>
<td>1.66</td>
<td>16.89</td>
<td>+0.04</td>
<td>82.7</td>
<td>58.8</td>
</tr>
<tr>
<td>72</td>
<td>16.36</td>
<td>14.42</td>
<td>2.02</td>
<td>16.44</td>
<td>+0.08</td>
<td>46.1</td>
<td>145.9</td>
</tr>
<tr>
<td>78</td>
<td>15.53</td>
<td>13.66</td>
<td>2.09</td>
<td>15.75</td>
<td>+0.22</td>
<td>32.4</td>
<td>345.0</td>
</tr>
<tr>
<td>96</td>
<td>14.39</td>
<td>12.66</td>
<td>2.63</td>
<td>15.29</td>
<td>+0.90</td>
<td>11.2</td>
<td>269.6</td>
</tr>
<tr>
<td>120</td>
<td>13.02</td>
<td>11.47</td>
<td>2.53</td>
<td>14.00</td>
<td>+0.98</td>
<td>4.9</td>
<td>226.4</td>
</tr>
</tbody>
</table>

Table 3.15: Average carbon data obtained from the MEP(10mM PO₄³⁻) cultures described in Section 3.6.2. 
C_w = carbon in whole broth 
C_m = carbon in medium only 
C_b = carbon in biomass

<table>
<thead>
<tr>
<th>TIME (h)</th>
<th>N_w (g·L⁻¹)</th>
<th>N_m (g·L⁻¹)</th>
<th>N_b (g·L⁻¹)</th>
<th>N_m+N_b (g·L⁻¹)</th>
<th>DIFFERENCE OF N_m+N_b FROM N_w (mg·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.18</td>
<td>2.10</td>
<td>0.10</td>
<td>2.20</td>
<td>+0.02</td>
</tr>
<tr>
<td>48</td>
<td>2.14</td>
<td>1.69</td>
<td>0.46</td>
<td>2.15</td>
<td>+0.01</td>
</tr>
<tr>
<td>54</td>
<td>2.14</td>
<td>1.53</td>
<td>0.66</td>
<td>2.19</td>
<td>+0.05</td>
</tr>
<tr>
<td>72</td>
<td>2.12</td>
<td>1.43</td>
<td>0.75</td>
<td>2.18</td>
<td>+0.06</td>
</tr>
<tr>
<td>78</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>96</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>120</td>
<td>2.10</td>
<td>1.33</td>
<td>0.94</td>
<td>2.27</td>
<td>+0.17</td>
</tr>
</tbody>
</table>

Table 3.16: Average nitrogen data obtained from the MEP(10mM PO₄³⁻) cultures described in Section 3.6.2. ND=no data. 
N_w = nitrogen in whole broth 
N_m = nitrogen in medium only 
N_b = nitrogen in biomass
Figure 3.109: Graph showing biomass (●-○-) and deoxyribose (■-□-) changes during two cultures (flask 1, solid symbols; flask 2, hollow symbols) grown in MEP medium with a 10mM phosphate supplement.

Figure 3.110: Graph showing changes in selected carbon sources, glucose (■-□-), maltose (●-■-) and glycerol (■-□-) during two cultures (flask 1, solid symbols; flask 2, hollow symbols) grown in MEP medium with a 10mM phosphate supplement.
Figure 3.111: Graph showing changes in organic acids, pyruvate (●○-) and α-ketoglutarate (▲●) during two cultures (flask 1, solid symbols; flask 2, hollow symbols) grown in MEP medium with a 10mM phosphate supplement.

Figure 3.112: Changes in pH during two cultures (flask 1, ---; flask 2, ...) grown in MEP medium with a 10mM phosphate supplement.
Figure 3.113: Graph showing average changes in carbon content of a culture (total carbon – ■–, carbon in medium – ●–, biomass – ◦– and CO\textsubscript{2} – ○–) and the proportion of consumed carbon passing to organic acids, pyruvate (– ▲–) and α-ketoglutarate (– ▼–) during cultures grown in MEP medium with a 10mM phosphate supplement.

Figure 3.114: Graph showing average changes in nitrogen content (total nitrogen – ■–, lost nitrogen – ○–, nitrogen in medium – ●– and biomass – ◦–) during cultures grown in MEP medium with a 10mM phosphate supplement.
Figure 3.115a: Diagrammatic representation of the fate of carbon consumed by 48h by cultures grown in MEP medium with a 10mM phosphate supplement. The initial carbon at 0h was 18.34g.L⁻¹. Values in bold show the percentage of the carbon consumed (indicated on the left) which passed to biomass, excreted acids and carbon dioxide.
Figure 3.115b: Diagrammatic representation of the fate of carbon consumed by 72h by cultures grown in MEP medium with a 10mM phosphate supplement.
96h
CARBON SOURCE CONSUMED FROM MEP(10mM PO₄³⁻)
MEDIUM SINCE TIME 0h:
5.96g.L⁻¹ (32.50%)

RESIDUAL CARBON SOURCE:
12.38g.L⁻¹ (67.50%)

BIOMASS CARBON INCREASE SINCE 0h:
2.30g.L⁻¹

% OF TOTAL CARBON CONSUMED SINCE 0h:
38.6

METABOLISM:
carbohydrate and amino acid carbon source

GLYCOLYSIS

pyruvate

TCA CYCLE

α-ketoglutarate

CO₂

Carbon content of excreted pyruvate:
0.012g.L⁻¹

Carbon content of excreted ketoglutarate:
0.269g.L⁻¹

Figure 3.115c: Diagrammatic representation of the fate of carbon consumed by 96h by cultures grown in MEP medium with a 10mM phosphate supplement.
Figure 3.115d: Diagrammatic representation of the fate of carbon consumed by 120h by cultures grown in MEP medium with a 10mM phosphate supplement.
3.7 Carbon and Nitrogen Limitation and Effect of Carbon Source on Growth Rate

3.7.1 Carbon and Nitrogen Limitation in Defined Media

A series of experiments were conducted to investigate the range of carbon and nitrogen limitation of *S. lividans* TK24 in two of the simplest defined media, glucose-ammonium and glucose-nitrate. Differences in growth in these media have been described in Section 3.2.2.1.

A range of carbon:nitrogen (C:N) ratios between 3:1 and 20:1 were investigated, summarised in Table 3.17.

<table>
<thead>
<tr>
<th>C:N RATIO</th>
<th>INITIAL NITRATE (mM)</th>
<th>INITIAL AMMONIUM (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:1</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>6:1</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>9:1</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>12:1</td>
<td>15</td>
<td>7.5</td>
</tr>
<tr>
<td>15:1</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>20:1</td>
<td>9</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Table 3.17: C:N ratios used to investigate carbon and nitrogen limitation in glucose-nitrate or glucose-ammonium media. The initial glucose concentration was kept consistent at 30mM; nitrate and ammonium were varied as shown. Ammonium supplies twice as much nitrogen as nitrate.*

Six shake flask cultures were investigated, covering the whole range of ratios in glucose-nitrate media; and a further six investigated for glucose-ammonium media. Each experiment was performed once. Each culture was grown for 72h, over which the biomass levels were recorded as DCW (g·L⁻¹). The results are presented in Figures 3.116 and 3.117.

From both Figures 3.116 and 3.117, it can be seen that in the region of C:N ration 20:1 to 12:1 the cultures were nitrogen limited. Biomass levels plateaued above a ratio of 9:1, indicating carbon limitation. Glucose-ammonium cultures were apparently more sensitive to carbon limitation. It can be deduced that the critical C:N ratio for both kinds of media is approximately 10:1. At this point the cultures should be neither carbon or nitrogen limited.
Figure 3.116: Graph showing carbon and nitrogen limitation regions in glucose-nitrate media. The initial glucose concentration was 30mM in all media. C:N ratios corresponding to initial nitrate concentrations are given in Table 3.17.

Figure 3.117: Graph showing carbon and nitrogen limitation regions in glucose-ammonium media. The initial glucose concentration was 30mM in all media. The C:N ratios corresponding to initial ammonium concentrations are given in Table 3.17.
Other glucose defined media summarised in Table 3.6 presented C:N ratios of between 5:1 and 17:1. Media 8-11 (glucose with mixed nitrogen sources) contained initial C:N ratios of between 5 and 6:1, indicating some carbon limitation. Media 1 and 2 contained ratios of 6:1, again indicating some carbon limitation. This may reflect the fact that glucose was exhausted but nitrate was not from glucose-nitrate cultures. (Figure 3.6). However, the fact that neither glucose or ammonium were exhausted from other glucose-inorganic nitrogen source cultures (Figure 3.4) indicates alternative limitation of, for example, intracellular effectors or other factors in the defined SMM medium.

Media 3-7 (glucose-amino acid media) presented C:N ratios of between 9:1 and 17:1. Glucose-lysine medium, at a ratio of 9:1, may have been balanced for carbon and nitrogen. Other media may have experienced nitrogen limitation, although carbon and nitrogen were completely exhausted from these media.

It should also be noted that actual biomass yield and growth performance appear to be dependent on the specific nitrogen source. From Figures 3.116 and 3.117 it can be seen that even at the critical C:N ratio the biomass yield from glucose-ammonium medium was half that in glucose-nitrate medium. The yield of biomass per gram of consumed glucose was less than half in comparison.

In glucose-mixed nitrogen source media (8-11 in Table 3.6), the maximum biomass yield was lower in alanine media which had initial C:N ratios of 5:1 compared to aspartate media, where the C:N ratio (5.5:1) was marginally closer to the critical point. This might seem to fit a reasonable hypothesis relating biomass yield to limitation. However, in glucose-amino acid media, the biomass yield was generally greater in those media with C:N ratios furthest from the critical point (e.g. glucose-aspartate and glucose-glutamate at 16:1 and 17:1 respectively). The biomass yield was lower in media with less nitrogen limitation where the C:N ratio was closer to the critical point (glucose-alanine and glucose-glycine at 15:1 and 14:1 respectively). The biomass yield was the poorest in glucose-lysine medium which would have been thought to have the best C:N balance (9:1).

Of course, regarding the specific effects of the nitrogen source mentioned previously, it may be that the critical point varies with other nitrogen sources, for example between organic and inorganic types. It is unfortunate that time constraints prevented further investigation of this factor.
In defined media with alternative carbon sources to glucose the C:N ratios tended to be identical. In starch media the ratios could not be calculated exactly. The initial carbon was however measured in media with starch and nitrate, alanine or aspartate and was found to be identical to the equivalent glucose media. C:N ratios in nitrate or aspartate media with maltose, glycerol or fructose were identical to glucose types at 6:1 or 16:1 respectively. Again, variations in biomass yield (compare Tables 3.12 and 3.8) may suggest extra differences caused by the specific carbon source on the critical C:N ratio.
3.7.2 Specific Growth Rates in Defined Media

Maximum specific growth rates ($\mu_{\text{max}}$) were calculated for each of the defined cultures presented in Sections 3.2 to 3.4. Values were calculated from samples taken every 6h from 120h cultures. This data is useful for a true indication of which carbon sources were utilised most rapidly by *S. lividans* TK24. The average $\mu_{\text{max}}$ for each carbon source are shown in Table 3.18.

<table>
<thead>
<tr>
<th>CARBON SOURCE</th>
<th>RANGE OF $\mu_{\text{max}}$ (h$^{-1}$)</th>
<th>AVERAGE $\mu_{\text{max}}$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>0.036-0.081</td>
<td>0.053 ± 0.0141</td>
</tr>
<tr>
<td>starch</td>
<td>0.040-0.063</td>
<td>0.048 ± 0.0074</td>
</tr>
<tr>
<td>maltose</td>
<td>0.039-0.043</td>
<td>0.041 ± 0.0043</td>
</tr>
<tr>
<td>glycerol</td>
<td>0.022-0.039</td>
<td>0.031 ± 0.0085</td>
</tr>
<tr>
<td>fructose</td>
<td>0.018-0.037</td>
<td>0.028 ± 0.0095</td>
</tr>
<tr>
<td>complex</td>
<td>0.041-0.095</td>
<td>0.064 ± 0.0050</td>
</tr>
</tbody>
</table>

Table 3.18: Maximum specific growth rates on various carbon sources.

For the complete range of glucose defined media, $\mu_{\text{max}}$ varied between 0.004 and 0.097h$^{-1}$, with an average value of 0.042. The lowest $\mu_{\text{max}}$ was recorded in glucose-ammonium and glucose-lysine media, improving through glucose-alanine-ammonium, glucose-alanine-nitrate and glucose-nitrate media. Values near the average were recorded in glucose-aspartate-nitrate, glucose-alanine, glucose-glycine and glucose-glutamate media. The highest $\mu_{\text{max}}$ were found in glucose-aspartate and glucose-aspartate-ammonium media. Considering just the nitrogen sources which were used in later media (nitrate, alanine, aspartate) the range of $\mu_{\text{max}}$ gave an average of 0.053h$^{-1}$.

The average $\mu_{\text{max}}$ for other carbon sources decreased in the order starch-maltose-glycerol-fructose. Although it should be noted that certain carbon sources were used in only two types of media, this pattern fits with the starch-maltose-glycerol order of preference reported in cultures of *S. coelicolor* by Liao *et al.* (1995). The $\mu_{\text{max}}$ was generally greater in media with amino acids compared to nitrate.

The average $\mu_{\text{max}}$ was also calculated for complex media where carbon was supplied as malt extract or malt extract broth. This was higher than defined media at 0.064h$^{-1}$, which would perhaps be expected considering the generally enhanced biomass yield in most of these media compared to defined types (compare Tables 3.8, 3.12 and 3.14).
3.8 Stirred Vessel Fermentations

3.8.1 Objective

The shake flask cultures described in Section 3.2-3.5 enabled the collection of data about the growth of \textit{S. lividans} and the excretion of organic acids by this organism. The aim of this work was to then complete a series of stirred vessel fermentations in selected media to see if acid identities and production profiles were the same as in shake flasks; to see if \(\alpha\)-ketoglutarate was reused as a carbon source during fermentations under controlled conditions, and to see if ammonium ions had the same effect (to prevent acid over production) during growth in a more controlled environment. Data for fermentations in a medium supporting acid production (glucose-aspartate) without pH control and in a medium not supporting acid production (glucose-aspartate-ammonium) are presented in this section.
3.8.2 Results

Data from a 2L stirred vessel fermentation of *S. lividans* TK24 in SMM medium containing glucose and aspartate is shown in Figures 3.118-3.120. Due to the failure of the air controller after 72h, this fermentation was not run for the 96h aimed for, so it is not possible to say if any more α-ketoglutarate was reused than in shake flasks. From Figure 3.118 it can be seen that the biomass profile for this fermentation was comparable to the equivalent shake flask cultures (Figure 3.17). The maximum recorded biomass was 2.1g·L⁻¹ DCW at 48h and it was expected that the biomass would peak again at around 78h. Other growth parameters are shown in Figure 3.120. The levels of biomass in the broth were lower, apparently due to the deposition of a large amount of material on the glass of the vessel above the liquid level. This problem was encountered due to rapid foaming between periods of active foam control in this fermentation. The material was in the form of dispersed mycelia with some small pellets, although less in number and smaller in size than in shake flasks.

As in shake flasks, aspartate and glucose were exhausted by 48-54 and 72h respectively (Figure 3.119), corresponding to the (two) peaks in biomass. Organic acids were detected in the broth from 24-30h. Again, only pyruvate and α-ketoglutarate were detected. Levels increased throughout the remainder of the fermentation to maxima of 250.1mg·L⁻¹ and 673.5mg·L⁻¹ respectively. Concentrations may have been lower due to the reduced biomass levels in the broth. However, at the maximum points, pyruvate represented 3.4% and α-ketoglutarate 9.2% of the consumed carbon, which was comparable to glucose-aspartate shake flask cultures. From Figure 3.120 it can be seen that the pH of the culture rose to around 7.4 at 50h (as would be expected from the rapid utilisation of the amino acid in this period; Bader, 1986) and fell sharply between 50 and 66h to pH 5.5 during rapid acid excretion. After 66h, the pH began to rise suggesting the reuse of some of the acids. Alternatively this may be related to the low DOT at this point. From the fermentations conducted in which acids were produced (incomplete data not presented) it appears that the fall in DOT is a characteristic event during rapid biomass accumulation and acid excretion.

Data from a 2L stirred vessel fermentation of *S. lividans* in SMM medium containing glucose, aspartate and ammonium is shown in Figure 3.121-3.123. As with previous defined medium fermentations, the profile of biomass accumulation (Figure 3.121) was fairly comparable to shake flask fermentations (Figure 3.29) with the peak
Figure 3.118: Graph showing biomass changes during a 2L stirred vessel fermentation in SMM medium containing 30mM glucose and 15mM aspartate.

Figure 3.119: Graph showing changes in carbon and nitrogen sources, glucose (■), and aspartate (▲), and organic acids, pyruvate (●) and α-ketoglutarate (▲) during a 2L stirred vessel fermentation in SMM medium containing 30mM glucose and 15mM aspartate.
Figure 3.120: Fermentation data (pH, dissolved oxygen tension, oxygen uptake rate and CO₂ evolution rate) from a 2L stirred vessel fermentation in SMM medium containing 30mM glucose and 15mM aspartate.

Figure 3.121: Graph showing biomass (●) and deoxyribose (▼) changes during a 2L stirred vessel fermentation in SMM medium containing 30mM glucose, 15mM aspartate and 15mM ammonium.
Figure 3.122: Graph showing changes in carbon and nitrogen sources, glucose (■) and aspartate (▲) during a 2L stirred vessel fermentation in SMM medium containing 30mM glucose, 15mM aspartate and 15mM ammonium. Ammonium not shown but present throughout.

Figure 3.123: Fermentation data (pH—, dissolved oxygen tension———, oxygen uptake rate— and CO₂ evolution rate ——) from a 2L stirred vessel fermentation in SMM medium containing 30mM glucose, 15mM aspartate and 15mM ammonium.
recorded at 48h. Levels were again lower (1.5g·L⁻¹ DCW compared to 3.3g·L⁻¹) due to the deposition of material on the vessel walls. Other growth parameters are shown in Figure 3.123. Glucose utilisation occurred at a similar rate to the equivalent shake flasks and this substrate was exhausted by 48h (Figure 3.122). The presence of aspartate until 72h may explain the continuation of replicatory growth until this time (DNA analysis Figure 3.121). Ammonium was present throughout. From Figure 3.123 it can be seen that the pH of this culture did not fall significantly and no acids were detected by HPLC. Therefore in fermentations as in shake flask cultures, ammonium appeared to prevent acid excretion completely. Carbon analysis of samples from this fermentation showed that approximately 75.4% of the consumed carbon was lost to CO₂ through respiration. When compared to the 64.5% estimated loss of carbon to CO₂ from glucose-aspartate shake flask cultures, it can be concluded that the excretion of organic acids reduces the volume of carbon passing through aerobic energy-generating metabolic pathways, a by-product of which is CO₂. This would suggest that in acidifying cultures less energy and metabolic precursors are supplied by the central metabolic pathways and hence biomass is less than it might otherwise be. However, the addition of ammonium to cultures does not always increase the biomass yield, as mentioned in previous sections. These hypotheses will be discussed in more detail in Section 4.

The main conclusions which could be drawn from the fermentations conducted were that S. lividans TK24 did not grow any better or faster in a small-scale fermenter compared to shake flask cultures; that S. lividans TK24 produced exclusively pyruvic and α-ketoglutaric acids regardless of the vessel used for culture; and that ammonium ions could prevent acid excretion in shake flasks and small-scale fermenters (unpublished work indicates that at smaller shake flask scales, the organism is oxygen-limited and its metabolism is altered substantially such that ammonium no longer prevents acid production; Susan Robinson, personal communication).
4 DISCUSSION

4.1 Comparison of Organic Acid Production by Selected Streptomyces Species: Influence of Carbon and Nitrogen Sources

During this project, *Streptomyces lividans* TK24 was found to excrete the organic acids pyruvate and α-ketoglutarate in almost all media tested during shake flask cultures. Overproduction of these metabolites occurred in the presence of a rapidly-utilised carbon source (such as glucose, starch, maltose and glycerol, but excluding fructose). This observation extended to complex media whose components were found to provide a selection of these carbon sources; for example malt extract was found to be composed of approximately 50% maltose, 20% glucose and 30% other materials including the carbon sources sucrose and dextrin, which are not readily-utilised by *S. lividans* TK24.

Of the nitrogen sources tested, amino acids and nitrate supported acid excretion in defined media. This extended to complex media where peptone acted as a source of amino acids, or where amino acids and nitrate were added as additional nitrogen sources so long as a readily used carbon source was also available. Ammonium prevented or reduced acid excretion when it was present at a greater concentration than that derived naturally from the degradation of amino acids. In defined media, the presence of ammonium ions prevented the release of organic acids into the medium completely. In complex malt extract-peptone medium, which supplied a larger amount of carbon compounds supporting acid production, an ammonium supplement reduced the excretion of organic acids by around 85%. Finally, not only specific carbon sources but also organic nitrogen sources were found to contribute carbon to excreted organic acids, as shown in studies using radiolabelled substrates.

4.1.1 Influence of Carbon Source

*S. lividans* was found to excrete glycolytic and TCA cycle organic acids which are typical products of acidifying streptomycete fermentations (stated by Payne *et al.*, 1990). Cultures of *S. griseus*, *S. aureofaciens* and *S. alboniger* have been reported to excrete pyruvate in complex or defined media containing a rapidly-used carbon sources such as glucose or sucrose (Hockenhull *et al.*, 1954; Doskocil *et al.*, 1959; Surowitz & Pfister, 1985). Later authors such as Ahmed *et al.* (1984) and Dekleva & Strohl (1987)
reported the excretion of both pyruvate and α-ketoglutarate by cultures of *S. venezuelae* and *S. peucetius*. During this project, *S. lividans* TK24 was observed to produce large amounts of pyruvate and α-ketoglutarate. The common denominator in acid production by most of the *Streptomyces* strains reported and *S. lividans* TK24 is that they all excrete excessive amounts of pyruvate, except the *S. lividans* 66 example reported by Payne *et al.* (1990) and DelaCruz *et al.* (1992) which will be considered later in this section. Further details can be found in Section 1.2.1. Many of the later researchers also investigated the influence of the carbon and nitrogen sources employed on acid production. These results will be compared to those presented in this thesis.

Chatterjee & Vining (1982), Chatterjee *et al.* (1983) and Ahmed *et al.* (1984) reported the production of high levels of pyruvate and α-ketoglutarate by *S. venezuelae* in defined media containing glucose and ammonium sulphate. Acid production was less with other carbon sources including starch, fructose, maltose, glycerol and lactose, although no specific details were given. Chatterjee *et al.* (1983) found that *S. venezuelae* utilised glucose preferentially to lactose and that the organism grew better on glucose than lactose, even though glucose supported high acid production and lactose did not. During this project it was also found that growth of *S. lividans* on glucose resulted in greater acid production than other carbon sources, including starch, maltose and glycerol. Fructose did not support acid production. By comparing, for example, Figures 3.17/3.18 (glucose-aspartate) and 3.56/3.57 (starch-aspartate) to 3.64/3.65 (maltose-aspartate), 3.68/3.69 (glycerol-aspartate) and 3.72/3.73 (fructose-aspartate) it can be seen that glucose and starch were utilised more rapidly from defined media and supported greater biomass accumulation by *S. lividans* than the other carbon sources. Generally glucose and starch media also supported higher acid production. In complex media, supplying 'mixed' carbon sources as malt extract, it was commonly observed that glucose was used preferentially to the other major component of malt extract (maltose) or the added primary carbon source (glycerol). This was unexpected since it had been thought that glycerol would be favoured as the principal carbon source (Wrigley-Jones *et al.*, 1993). From Figure 3.75 sequential utilisation can be observed, with glucose then maltose utilised first between 24 and 48h. Subsequent rapid glycerol uptake was delayed until after glucose exhaustion at 48h and the apparent cessation of maltose uptake at this time. Liao *et al.* (1995) reported that in the closely related strain *S. coelicolor*, glycerol, maltose and starch supported increasingly faster and apparently
greater levels of growth in defined media with glutamate as a nitrogen source. Thus the preference for glucose/starch over maltose over glycerol is not restricted to *S. lividans*.

The results presented in Section 3.2 and 3.4 suggest that acid overproduction by *S. lividans* is greatest during growth on the most rapidly-utilised carbon sources (glucose and starch, Section 3.7.2). This may indicate that acid excretion occurs due to an overload of the central metabolic pathways with excess carbon source. This will be discussed further in Section 4.2. Comparison with *S. venezuelae* implies that this may be a common phenomenon in *Streptomyces* although this aspect has unfortunately not been studied intensively. Dekleva & Strohl (1987) did study the influence of carbon sources on acid production by *S. peucetius*, but did not report whether those substrates supporting acid production were used more rapidly or preferentially to those which did not. In defined medium containing glucose or fructose and nitrate, or complex medium with extra glucose, this organism excreted pyruvate and α-ketoglutarate. Cultures grown in nitrate-defined medium with starch, maltose or dextrin did not acidify. Surowitz & Pfister (1985) also found that *S. alboniger* produced pyruvate in glucose-based complex medium but not dextrin, glycerol or fructose-containing media. In contrast during this project, glucose, starch, maltose and glycerol were found to support acid production by *S. lividans*, while fructose did not. Dekleva & Strohl (1987) suggested that acid production was a carbon-source-specific effect. Therefore these apparent differences may reflect variations in carbon source metabolism between and perhaps within different strains. It was possible to calculate maximum specific growth rates of *S. lividans* TK24 for each carbon source, but it is unfortunate that the specific uptake rate of either carbon or nitrogen nutrients was not investigated due to time constraints. This would be an interesting aspect of *S. lividans* metabolism to study in order to investigate the relationship of acid excretion with uptake rate and uptake method of various carbon and nitrogen sources.

Finally, *S. peucetius* and *S. alboniger* also did not reassimilate organic acids while most of the other strains documented did (*e.g.* *S. venezuelae* and *S. coelicolor*). *S. lividans* apparently reused pyruvate to fuel biomass accumulation in some cultures (for example glucose-alanine cultures, Figures 3.9 and 3.10).

### 4.1.2 Influence of Nitrogen Source

While it seems that acid production by *S. lividans* is always supported by the most rapidly-utilised carbon sources, there is no overall trend apparent for nitrogen
sources. Few researchers have investigated the relationship of acid production and nitrogen sources, most choosing to restrict the nitrogen source used to one type (e.g. complex sources by Surowitz & Pfister, 1985; and nitrate by Dekleva & Strohl, 1987). However, the results obtained during this project for *S. lividans* TK24 can be compared again to *S. venezuelae* (Ahmed *et al.*, 1984).

In *S. lividans* cultures acid production occurred only in defined media containing nitrate salts or amino acids, or in complex media supplying nitrogen as a mixture of amino acids in the form of peptones. Organic acid excretion was prevented or reduced in defined or complex media containing ammonium salts. It would therefore be expected that the growth of *S. lividans* would be improved in media containing ammonium. It is thought that ammonium is more readily utilised as a nitrogen source than others such as amino acids and nitrate (Ahmed *et al.*, 1984; Demain, 1992). In cultures of *S. lividans* in defined media this was not generally true. The biomass yield in medium containing 30mM glucose and 15mM ammonium salt was lower (Figure 3.3) than the equivalent medium containing nitrate supplying an equal amount of nitrogen (Figure 3.5). The biomass yield was also low in glucose-lysine (Figure 3.25) and glucose-glycine (Figure 3.13) cultures, perhaps reflecting the complexity of lysine and the poor carbon content of glycine. Some of the best biomass yields in defined media were obtained with glucose-alanine (Figure 3.9), glucose-aspartate (Figure 3.17) and glucose-glutamate media (Figure 3.21). A similar relationship of growth to nitrogen source in defined medium was noted for the closely related strain *S. coelicolor* by Liao *et al.* (1995). With this strain, growth was poor on ammonium, nitrate and complex amino acid nitrogen sources including lysine. It was implied by these authors that nitrate was used extremely poorly as a nitrogen source, which is in keeping with the observations made during this project. Liao *et al.* (1995) also observed that *S. coelicolor* cultures supplied with simple amino acids (including glutamate) or, peculiarly, complex nitrogen sources such as peptone exhibited a higher growth rate. This again matches the results summarised above for *S. lividans* (refer to Figures 3.74 and 3.85 for improved growth on malt extract-peptone and peptone-nitrate media). Nitrate salts were present throughout all of the *S. lividans* TK24 cultures to which they were added (Sections 3.2.2.1, 3.2.2.3, 3.3.2.1, 3.4.2.1, 3.5.2.2 and 3.5.2.3), while amino acids were used to varying degrees of rapidity and completion, usually reflecting the biomass yield of the culture (for example Section 3.2.2.2). Ammonium salts also tended to be present throughout the course of
cultures to which they were added (Sections 3.2.2.1, 3.2.2.3, 3.2.2.4 and 3.5.2.4). Other potential carbon and nitrogen limitation factors are considered in Section 3.7.1.

Recently, Zhang et al. (1996) reported that cultures of *S. griseofuscus* also preferred glutamate as a nitrogen source to ammonium, indicating that ammonium is not always preferred as a nitrogen source by all micro-organisms. The addition of an ammonium supplement to MEP(10mM PO_4^-3) cultures did improve biomass yields slightly (compare Figures 3.74 and 3.106) although the increase was not great (5.8g.L^-1 compared to 6.0g.L^-1 at maxima) and growth was no faster.

In contrast to the observations made during this project, Ahmed et al. (1984) reported that acid production by *S. venezuelae* was high in defined medium containing ammonium, but only after ammonium was exhausted, which occurred very rapidly. It should be noted that in some test *S. lividans* cultures in defined media with glucose and amino acids, but reduced levels of ammonium (data not shown), ammonium was still not exhausted within 120h, the biomass yield was less and acids were not detected. Therefore it cannot be said whether acid excretion would commence after ammonium exhaustion from *S. lividans* cultures as it did in the *S. venezuelae* cultures. Ahmed et al. (1984) reported that acid production by *S. venezuelae* was highest in glucose-nitrate medium and again occurred only after nitrogen source exhaustion which occurred at around 48h. Nitrate was utilised very poorly by *S. lividans*, although acid production was still very high in glucose-nitrate medium. Organic acids were excreted by *S. venezuelae* after exhaustion of a rapidly-utilised amino acid such as glycine but during the assimilation of a more slowly-used type such as serine. Organic acids were excreted by *S. lividans* during assimilation of all types of amino acids although some were clearly preferred to other amino acids, ammonium and nitrate. That ammonium was preferred as a nitrogen source by *S. venezuelae* was demonstrated by the fact that it was exhausted before 24h, compared to other sources which were exhausted around 48h. Ammonium was also used sequentially before nitrate in a mixed nitrogen source culture.

The differences in the acid production-nitrogen source relationships between *S. lividans* and *S. venezuelae* are very great (particularly the role of ammonium) and may be explained by differences in nitrogen source metabolism in these strains. Ahmed et al. (1984) suggested that acid production by *S. venezuelae* occurred when cultures were nitrogen limited, either after depletion of rapidly-utilised types or during growth on limiting sources. This generalisation cannot be applied to *S. lividans* TK24. Acid excretion occurred during growth of *S. lividans* on both rapidly-utilised amino acid
nitrogen sources (e.g. Figures 3.10, 3.14, 3.18 and 3.22; note how pyruvate was excreted and reused before amino acid depletion) and on poorly-utilised amino acids (Figures 3.26 and 3.38) and nitrate (Figure 3.6) but not on poorly-utilised ammonium. In addition, even the generalisation made by Ahmed et al. (1984) is not absolute. These workers and Surowitz & Pfister (1985) noted that proline, by all accounts a very slowly-used amino acid (Demain, 1992), did not support high organic acid production.

A tentative conclusion can be suggested where organic acid over-production by *S. lividans* does not occur when ammonium is available, but does occur when excess ammonium is not available. However, depletion of ammonium from alanine-ammonium cultures did not induce acid production, presumably due to the absence of glucose. Of course it may still be construed that acid production occurred during nitrogen limitation if both amino acids and nitrate are regarded as limiting sources of nitrogen and ammonium as non-limiting (suggested by Demain, 1992). However, indications were that amino acids were preferred by *S. lividans* to ammonium. The potentially specific metabolic requirements of *S. lividans* for ammonium will be discussed in Section 4.2.

### 4.1.3 Variations in Acid Production Profiles of *Streptomyces* Species

Certain similarities are evident in the organic acid production profiles of *S. lividans* (this project) and *S. venezuelae* (where shown in the article of Ahmed et al., 1984). In both strains pyruvate levels tend to peak and then fall rapidly, often to exhaustion. α-Ketoglutarate levels peak and remain high throughout the rest of the culture. A similar profile was reported by Hobbs et al. (1992) for the closely related strain *S. coelicolor* during a stirred vessel culture in defined medium containing around 60mM glucose and 30mM alanine. It is notable that in their medium, which is very similar to that described in Section 3.2.2.2, acid levels peaked at the same times (pyruvate at 54h and α-ketoglutarate at 72h) and the maximum concentrations were similar (pyruvate around 215mg-L⁻¹ and α-ketoglutarate about 270mg-L⁻¹, compared to 330mg-L⁻¹ and 415mg-L⁻¹ respectively in the *S. lividans* culture). Again pyruvate was reused rapidly by *S. coelicolor* while α-ketoglutarate levels remained high.

Nitrogen sources were exhausted very rapidly during many of the *S. venezuelae* cultures reported by Ahmed et al. (1984), causing some of the nitrogen limitation which these authors believed led to acid excretion. Hobbs et al. (1992) did not report whether alanine was depleted from their medium, but it is possible that it was exhausted during the 144h course of the culture. These other reports indicate that (i) any nitrogen
limitation occurring in \textit{S. lividans} cultures is not uncommon to streptomycete cultures in defined medium; and (ii) \(\alpha\)-ketoglutarate does not tend to be reused to any great degree by a number of \textit{Streptomyces} strains, whether nitrogen-limited or not. Obanye \textit{et al.} (1996) have suggested that pyruvate is reassimilated by \textit{S. coelicolor} for incorporation into antibiotic products, or for gluconeogenesis during glucose starvation. Results from \textit{S. lividans} indicate that the latter is not always the case, since pyruvate may be reused while glucose is still present (\textit{e.g.} glucose-alanine medium, Figure 3.10; glucose-glutamate medium, Figure 3.22; and glucose-lysine medium, Figure 3.26).

The similar preference for carbon and nitrogen sources by \textit{S. lividans} (this project) and \textit{S. coelicolor} (Liao \textit{et al.}, 1995) have been noted previously in this section. It is worth noting that in neither the 1995 report of Liao, Vining & Doull nor the 1990 reports by Doull & Vining was acid excretion reported. In this work, \textit{S. coelicolor} was grown in defined medium containing starch and glutamate, or other carbon and nitrogen sources which would have supported acid excretion by \textit{S. lividans}. It can be assumed that acid production was prevented or buffered by the addition of MOPS buffer (morpholinopropanesulphonic acid) to the medium. This method was originally used by Aharonowitz & Demain (1977) to control pH fluctuations by \textit{S. clavuligerus}, and later by Chatterjee & Vining (1982), Chatterjee \textit{et al.} (1983) and Shapiro & Vining (1983). The prevention of acid excretion by \textit{S. lividans} TK24 will be discussed in Section 4.4.

DelaCruz \textit{et al.} (1992) reported that the organic acids excreted by cultures of \textit{S. lividans} 66 in complex medium containing glucose and tryptone were not pyruvate or \(\alpha\)-ketoglutarate, but isovalerate and acetate. This is in sharp contrast to the findings of this project for \textit{S. lividans} TK24, a derivative of strain 66. DelaCruz \textit{et al.} (1992) suggested that isovalerate was produced as a by-product of the degradation of leucine from tryptone, under conditions of glucose excess (refer to Section 1.2.1 for details). It is odd that no pyruvate or \(\alpha\)-ketoglutarate were detected but such high concentrations of isovalerate were recorded, given the high glucose concentration and the general similarity of tryptone to the peptone used during this project. According to the Oxoid Manual (5th edition, 1982), leucine is not the major component of tryptone. Instead glutamate is the greatest amino acid by weight (17.3%) and the carbon skeletons of this compound are ultimately converted to \(\alpha\)-ketoglutarate. In MEP medium, \(\alpha\)-ketoglutarate production by \textit{S. lividans} TK24 was very high and might have been related to the similarly high proportion of glutamate in bacto- and myco-peptone (8.8 and 12.1%
respectively). No isovalerate or acetate were detected in the culture medium of the TK24 shake flask fermentations conducted during this project.

It should be noted that glucose was never in excess but was exhausted rapidly from defined *S. lividans* TK24 cultures. Excess glycerol rather than glucose was used as the primary carbon source in complex media. It can be speculated that if glucose had been in excess in either defined or complex cultures of *S. lividans* TK24, that acidic by-products of amino acid degradation may have been detected in the extracellular medium. This is predicted by the first part of the DelaCruz *et al.* (1992) hypothesis (described in Section 1.2.1), used by these authors to explain isovalerate excretion by *S. lividans* 66. However, results from *S. lividans* do not follow even the reverse of the DelaCruz model, where under low glucose concentrations amino acids are utilised to supply carbon leading to ammonium release and pH rise. What would be regarded as low glucose concentrations under the conditions of the model did not lead to pH increase, but still supported pyruvate and α-ketoglutarate production by *S. lividans* TK24. It was known that amino acids were used to supply carbon since radiolabelled studies (Section 3.3) showed that carbon from amino acids passed to excreted acids. In acidifying *S. coelicolor* A3(2) cultures, Tough & Prosser (1996) did observe that during rapid glucose utilisation the ammonia concentration rose in minimal medium. This was probably due to the use of casamino acids as the nitrogen source. However, these authors instead observed that the pH decreased, presumably due to α-keto acid release, as observed by Hobbs *et al.* (1992) in the same strain under similar conditions. The ammonium concentration rose towards the end of the acid production period which apparently ended after glucose exhaustion. Therefore, it cannot be said if the resulting high ammonium concentration affected organic acid production by *S. coelicolor* as it appears to in *S. lividans* TK24 cultures.

It is unlikely that the metabolism of *S. lividans* strains 66 and TK24 differ greatly. The addition of extra ammonium to acidifying media did prevent the pH fall as predicted by DelaCruz *et al.* (1992), although probably not for the reasons they suggested. Extra ammonium was also observed to prevent pH decreases during the growth of TK24 in YEME medium (Erpicum *et al.*, 1990). These authors suggested that extra ammonium prevented amino acid utilisation and implied (like DelaCruz *et al.*, 1992) that pH decrease was due to the excretion of amino acid carbon skeletons. Results from this project have indicated that TK24 utilised amino acids more readily than ammonium from defined media. It is possible instead that pyruvate and α-ketoglutarate
were excreted, and that ammonium prevented this excretion, during the studies of Erpicum et al. (1990). If ammonium can specifically prevent excessive organic acid production by \textit{S. lividans} TK24, then a new hypothesis to that of DelaCruz et al. (1992) is needed. This will be discussed further in Section 4.2.

The results of this project have confirmed that the pH decreases observed by Wrigley-Jones (1991) during \textit{S. lividans} TK24 cultures in MEP medium were due to the excretion of organic acids. The identities of the acids have been confirmed as pyruvate and $\alpha$-ketoglutarate. These are typical products of streptomycete cultures supplied with a rapidly-utilised carbon source such as glucose. It is apparent that organic acids are reassimilated by \textit{S. lividans}, but that in MEP medium this is not due to the exhaustion of the primary carbon source glycerol, as suggested previously (Wrigley-Jones et al., 1993). It is entirely possible that pyruvate was as readily used as a carbon source as others including glucose and maltose (e.g. Figures 3.12 and 3.22), but $\alpha$-ketoglutarate was not reused as readily as pyruvate. It is of course also possible that $\alpha$-ketoglutarate continued to be released at high levels in stationary phase, giving the appearance of low reuse during the cultures reported in Section 3. This is unlikely, since the primary carbon sources were exhausted before stationary phase in defined media, leaving nothing to fuel continued $\alpha$-ketoglutarate overproduction. Wrigley-Jones (1991) suggested that organic acids were excreted during the exponential phase and reused by \textit{S. lividans} in the transition to stationary phase. Results from this project showed that pyruvate was generally reused during rapid growth (e.g. Figures 3.9/3.10) while $\alpha$-ketoglutarate was reused well into the stationary phase (e.g. Figures 3.17/3.18).

Wrigley-Jones (1991) suggested that acid excretion occurred due to a metabolic imbalance such that glycolytic activity exceeded that of the TCA cycle. This is feasible since pyruvate and $\alpha$-ketoglutarate production generally occurred during the most rapid utilisation of carbon source in each acidifying culture. This is clear in Figure 3.8 for glucose-nitrate medium; Figure 3.12 for glucose-alanine medium (and other glucose-amino acid media); Figures 3.36 and 3.40 for glucose-amino acid-nitrate media; Figure 3.59 and 3.63 for starch-amino acid media; clearly in Figures 3.67 and 3.71 for maltose- and glycerol-aspartate media; and also Figures 3.75/3.76 for MEP(10mM PO$_4^{3-}$) medium, as well as other complex media. Further specific metabolic considerations will be made in Section 4.2.
4.2 Why Are Pyruvate and α-Ketoglutarate Excreted By *Streptomyces lividans*?

4.2.1 Summary of Observations

It has been shown during this project that the excretion of organic acids by *S. lividans* TK24 is dependent on the presence of a rapidly-utilised carbon source like glucose (Section 3.2). Acids were not detected in those media without such a sugar carbon source (Section 3.2.2.4). Glucose is metabolised aerobically via glycolysis to the end product pyruvate. At first sight it seems sensible that in a medium containing glucose and a ‘permissive’ nitrogen source such as nitrate (Section 3.2.2.1), a large amount of pyruvate would be excreted if a blockage of some kind existed in metabolism at a point beyond pyruvate (for example, at pyruvate dehydrogenase, PDH). That a significant amount of α-ketoglutarate is also excreted indicates a further blockage at a point beyond this intermediate (for example, at α-ketoglutarate dehydrogenase, α-KDH). Influences on acid production (carbon/nitrogen sources, blockages, enzyme regulation etc.) will be considered in this section.

In glucose-amino acid media (Section 3.2.2.2) the general trend observed was that pyruvate levels were decreased (consistently to maxima of around 300mg·L⁻¹ in media containing alanine, glycine, aspartate or glutamate) while α-ketoglutarate levels increased, relative to glucose-nitrate medium. Glucose-lysine cultures were an exception to this rule since acid production was lower than in other glucose-amino acid media. This was probably related to the poor growth of the organism in this medium (Figure 3.25) and the poor use of available substrates (Figure 3.26). Since very little lysine was consumed it was likely that the organism was severely nitrogen-limited from the start of these cultures, although growth picked up after 54h. Glucose-lysine cultures will be excluded except where specified from the following general conclusions.

There seems to be no obvious reason why pyruvate levels should be less in glucose-amino acid media (around 300mg·L⁻¹) compared to glucose-nitrate media (around 470mg·L⁻¹) since glucose was present at the same concentrations and was used as rapidly in most of the glucose-amino acid media. It is possible that the metabolism of glucose by the organism was facilitated or in some way improved by the utilisation of amino acids as organic nitrogen sources rather than inorganic nitrate, or that this was
related to nitrogen limitation in these media. Ammonium would be transaminated or
deaminated from amino acids during catabolic processes. It is possible that the influence
of ammonium on acid over-production occurs during metabolism, and is caused by
either ammonium supplied exogenously as ammonium sulphate (in cultures lacking acid
excretion) or ammonium generated during amino acid catabolism. A control factor
involving ammonium may be common to the pathways involved in both ammonium
uptake and amino acid degradation.

Looking briefly at the pyruvate and α-ketoglutarate maxima recorded in glucose-
amino acid media compared to glucose-nitrate cultures (Table 3.8, Section 3.2.3), it
might be concluded that pyruvate overproduction occurs following glucose catabolism
via glycolysis; and that α-ketoglutarate levels are increased due to the input of carbon
from the amino acids (hypothesis 1). Deamination of amino acids yields not only
ammonium but also carbon skeletons which are ultimately fed into the central metabolic
pathways (Zubay, 1993; Stryer, 1995) as shown in Figure 4.1. Alanine carbon skeletons
pass directly to pyruvate and in glucose-alanine cultures, pyruvate and α-ketoglutarate
maxima were very similar (329 and 415mg-L⁻¹ respectively, Figure 3.10). It might be
expected that pyruvate levels would exceed α-ketoglutarate levels if the scheme above
were followed. Pyruvate levels were higher in this medium than in other glucose-amino
acid media (329mg-L⁻¹ compared to 290-300mg-L⁻¹). Glycine carbon skeletons can pass
ultimately but not directly to pyruvate and may be degraded via an alternative route
which does not end in pyruvate. In glucose-glycine medium, maximum pyruvate did
exceed α-ketoglutarate by a ratio of approximately 3:2 (294 and 198mg-L⁻¹ respectively,
Figure 3.14). Aspartate and glutamate carbon skeletons ultimately enter the TCA cycle
beyond pyruvate. In glucose-aspartate or glucose-glutamate cultures maximum α-
ketoglutarate levels accordingly exceeded pyruvate levels such that the pyruvate:α-
ketoglutarate ratios were 1:3 in glucose-aspartate and 1:2 in glucose-glutamate media
(Figures 3.18 and 3.22).

It can be concluded that in glucose-amino acid media (i) glucose contributed
carbon to the excretion of up to 300mg-L⁻¹ pyruvate; and (ii) the position of entry of
amino acid carbon skeletons affected the maximum levels of pyruvate and α-
ketoglutarate. Alanine and glycine supply less carbon mole for mole (3xC and 2xC
respectively) than aspartate or glutamate (4xC and 5xC respectively) which may explain
why pyruvate levels were not as high as might have been expected in media with alanine
and glycine. Perhaps if initial alanine and glycine concentrations had been increased so
Figure 4.1: Diagram showing the fate of carbon skeletons from catabolised amino acids (after Zubay, 1993; Stryer, 1995).
that they supplied the same amount of carbon on a molar basis as aspartate and glutamate, then the pyruvate levels would have exceeded both pyruvate levels in aspartate or glutamate-containing media, and \( \alpha \)-ketoglutarate levels in alanine and glycine-containing media. In glucose cultures with aspartate or glutamate, \( \alpha \)-ketoglutarate levels did exceed pyruvate levels as expected. Glutamate carbon skeletons enter the TCA cycle finally at \( \alpha \)-ketoglutarate, perhaps explaining the elevated levels of this acid. Aspartate carbon skeletons enter the TCA cycle at oxaloacetate and supply less carbon, therefore it may seem odd that \( \alpha \)-ketoglutarate levels exceed those in glucose-glutamate media. Perhaps this is related to the fact that the TCA cycle ‘turns’ in the direction oxaloacetate to \( \alpha \)-ketoglutarate and an increase in carbon number occurs as the cycle passes citrate (see Figure 1.2) so maybe by the time metabolism reaches the blockage after \( \alpha \)-ketoglutarate, potentially more carbon is there to be excreted (compared to the input of carbon from glutamate), explaining the elevated \( \alpha \)-ketoglutarate levels. Finally, transamination of aspartate to oxaloacetate requires \( \alpha \)-ketoglutarate but generates more glutamate, perhaps also increasing acid levels.

In glucose-lysine media, maximum \( \alpha \)-ketoglutarate levels also exceeded those of pyruvate, reflecting the final position of entry of carbon from lysine. Reduced acid excretion may be related to poor carbon source use from this medium (note the way in which acid levels increased when rapid glucose uptake began, Figure 3.26). It may also be that extra ammonium resulting during from lysine degradation (lysine contains an extra \(-\text{NH}_2\) group on its side chain) influenced organic acid build-up. Lysine degradation proceeds along a very complicated path, involving at least 8 steps, compared to the other amino acids used in this work.

The above hypothesis may be an over-simplification in light of the results of the radiolabelling experiments presented in Section 3.3. In labelled glucose-alanine cultures it was found that a greater amount of carbon from alanine passed into excreted acids than from glucose (Figure 3.46). In addition, rather than glucose carbon being excreted as pyruvate and amino acid carbon as \( \alpha \)-ketoglutarate, carbon from both substrates was excreted in both organic acids. The trends were roughly as expected since more \(^{14}\text{C}\) from glucose was excreted in pyruvate than \( \alpha \)-ketoglutarate, while more \(^{14}\text{C}\) from alanine was excreted in \( \alpha \)-ketoglutarate than pyruvate (Figure 3.46). It can be seen by comparing Figures 3.43 (\(^{14}\text{C}\) glucose-nitrate medium) and 3.46 (labelled glucose-alanine
combinations) that the contribution of carbon from glucose to excreted acids was reduced in the presence of alanine.

In labelled glucose-aspartate cultures acid levels were generally higher, but the contribution of carbon from aspartate was greatly diminished compared to alanine. Again carbon from both substrates passed to excreted organic acids; and again more carbon passed from glucose to pyruvate than \( \alpha \)-ketoglutarate, and more carbon from aspartate to \( \alpha \)-ketoglutarate than pyruvate. The contribution of \( ^{14} \)C from glucose (Figure 3.49) was greater than in glucose-alanine cultures, although less than in the glucose-nitrate culture. This may have been to offset the low contribution from aspartate. It should be noted that the biomass yield was poor in the culture containing labelled aspartate and aspartate was utilised more slowly, therefore the passage of carbon from aspartate to organic acids may have been less than in other glucose-aspartate cultures. It can be seen that aspartate was used more rapidly than glucose in these cultures (Figure 3.48) or than alanine in other cultures (Figure 3.45) and was exhausted by 48h while glucose and alanine were exhausted by 72h. Since the biomass was higher in glucose-aspartate cultures (Figure 3.47) than glucose-alanine cultures (Figure 3.44), the results might suggest that aspartate carbon was used more efficiently than alanine carbon during biomass accumulation; also that more carbon was channelled from glucose than the amino acid to excreted acids in the presence of aspartate.

Alternatively it may be that it is less likely for aspartate carbon to pass to pyruvate. That any aspartate carbon is converted to pyruvate is surprising since aspartate carbon skeletons enter metabolism below pyruvate. Oxaloacetate can be decarboxylated to pyruvate and \( \text{CO}_2 \) by oxaloacetate decarboxylase, but this enzyme is not present all bacteria (it is absent from \textit{E. coli} but present in pseudomonads; Saier, 1987). The enzyme is coupled to the active extrusion of sodium ions from cells. It thus serves an important cellular function for the generation of an \( \text{Na}^+ \) electrochemical gradient which can be used to drive solute uptake or for the synthesis of ATP during oxidative phosphorylation.

Although the regulation of gluconeogenesis is poorly understood in \textit{Streptomyces}, it is unlikely that oxaloacetate would be converted to pyruvate via the alternative action of gluconeogenesis since (i) the organism was not suffering glucose starvation during the conversion of aspartate carbon to pyruvate (Figures 3.48/3.49); (ii) bacteria tend to lack some of the required enzymes, such as PEP carboxykinase (Saier, 1987); and (iii) gluconeogenesis and glycolysis are reciprocally regulated so that they
are not both active at once (Stryer, 1995). It was known that the TCA cycle and presumably glycolysis were active due to active pyruvate and \( \alpha \)-ketoglutarate formation from glucose carbon (Figure 3.49). This might explain why relatively little carbon from aspartate was excreted as pyruvate.

The fact that \( \alpha \)-ketoglutarate can be derived from sugar carbon as well as amino acid carbon was indicated in maltose-aspartate cultures, amongst others. \( \alpha \)-Ketoglutarate levels increased during rapid maltose utilisation rather than aspartate uptake (Figure 3.65, 78 to 120h). Of course, aspartate was also exhausted by 78h and may have contributed carbon to \( \alpha \)-ketoglutarate after deamination. The fact that pyruvate was excreted during aspartate utilisation while maltose levels remained constant (Figure 3.65, 30 to 54h) is also odd. This trend was not apparent in glucose-amino acid cultures (e.g. Figures 3.10 and 3.18) since the rapid use of glucose began at the same time as amino acids in these media.

It can be concluded that an extension of hypothesis 1 is valid such that (i) glucose contributes carbon principally to pyruvate and then to \( \alpha \)-ketoglutarate following glycolysis and the subsequent passage of carbon into the TCA cycle; (ii) amino acids contribute carbon principally to \( \alpha \)-ketoglutarate but also to pyruvate following the entry of carbon skeletons to the TCA cycle at pyruvate or below; and (iii) that metabolism varies depending on the specific carbon source, whereby some sources are utilised more rapidly than others and seemingly more efficiently for biomass accumulation than organic acid excretion (hypothesis 2). For example, aspartate is known to be involved in the biosynthesis of other amino acids from TCA cycle intermediates (Stryer, 1995).

The addition of nitrate salts to glucose-aspartate media (Section 3.2.2.3) caused a peculiar anomaly whereby the acid production profile (Figure 3.34) resembled that as if the amino acids were absent (Figure 3.6). Pyruvate levels exceeded \( \alpha \)-ketoglutarate levels and the maxima occurred simultaneously. The rapid uptake of glucose was delayed and that of aspartate was prolonged compared to glucose-aspartate medium (compare Figures 3.18 and 3.34). The simultaneous exhaustion of both substrates led to a single biomass peak, rather than the characteristic double peak of glucose-aspartate cultures. It is possible that nitrate was preferred to aspartate (unlikely since nitrate was very poorly used from all other cultures), or that nitrate uptake or metabolism somehow interfered with that of the other carbon and nitrogen sources. Alternatively it might be because these mixed nitrogen source media were less nitrogen limited than glucose-
amino acid media (Section 3.7.1). If nitrate did interfere with aspartate metabolism, then this might explain (i) why α-ketoglutarate levels were lower (in fact precisely the same as the glucose-nitrate cultures), (ii) why pyruvate levels were higher and (iii) why the biomass yield was reduced. In respect of point (ii) the conclusion might be that the metabolic blockage beyond pyruvate was more sensitive to an inorganic nitrogen source, since metabolism appeared to be principally blocked at that point (see Section 4.2.2).

The acid profile was similar in glucose-alanine-nitrate cultures with simultaneous maxima and pyruvate levels exceeding α-ketoglutarate levels (Figure 3.38). This again pointed to nitrogen source (nitrate) interference/interaction since the profiles of glucose-alanine (Figure 3.10) and glucose-aspartate cultures (Figure 3.18) were so disparate. In addition, the utilisation of alanine was reduced (also seen in glucose-alanine-ammonium cultures) with resultant poor biomass yields and lower acid production, and it is possible that these two are connected. The assumption that aspartate is favoured over alanine as a substrate for biomass accumulation is supported by the fact that aspartate was still rapidly utilised from media containing a competitive nitrogen source (e.g. Figures 3.30 with ammonium and 3.34 with nitrate) compared to alanine-containing cultures (Figures 3.32 with ammonium and 3.38 with nitrate).

The role of nitrate in *Streptomyces* metabolism is not well documented although the metabolism of this substrate proceeds down different pathways to that of ammonium or amino acids. It is known that in *E. coli* nitrate is reduced to nitrite by nitrate reductase, and nitrite is converted to ammonium by NADH-nitrite oxidoreductase. This process occurs under anaerobic conditions and nitrate is not used aerobically (Lin & Kuritzkes, 1987). Results from this project indicate that *Streptomyces* are capable of using nitrate as a nitrogen source during aerobic growth. This suggests differences in nitrogen metabolism regulation between these bacteria. In the originally soil-dwelling streptomycetes, it may be that inorganic nitrogen sources interact with and have 'dominant' effects on organic nitrogen sources. This might be an adaptation to their natural environment, where inorganic nitrogen sources may be more readily available than organic types.

All of the alternative carbon sources tested (starch, maltose, glycerol and fructose) enter glycolysis at a point above pyruvate (see Figure 1.1). It would therefore be no surprise if all of them supported acid overproduction. That fructose did not may
reflect the very poor use of this carbon source by \textit{S. lividans} TK24 (Figures 3.55 and 3.73; also Section 3.7.2). This may reflect a blockage or deficiency in fructose metabolism, perhaps at the step catalysed by fructokinase (fructose to fructose-6-phosphate). Good biomass yields in fructose-aspartate medium (Figure 3.72) were probably due to the utilisation of aspartate and again indicate the importance of this substrate for biomass formation.

Starch and maltose are composed of glucose monomers and it was expected that these would support acid production in defined media. In nitrate-based media organic acids were detected, but at low levels compared to glucose-nitrate cultures. This perhaps reflects the extra steps required in the catabolism of these sources or the presence of nitrate (other reasons are considered in Sections 4.2.2 and 4.3). Biomass yields were improved in both media even though maltose was not completely consumed, suggesting more efficient conversion of carbon to biomass. It has already been noted that the starch used in this project contained a very small proportion of glucose, and this may have induced a little extra acid production compared to maltose cultures.

The levels of acids detected in starch-amino acid media were very similar to glucose-amino acid media. Starch was always utilised rapidly indicating that it was as good a carbon source as glucose when compared to other carbon sources. In starch-amino acid media the organic acid maxima were slightly lower: 280mg-L^{-1} for pyruvate and 300mg-L^{-1} for \(\alpha\)-ketoglutarate in starch-alanine medium (compared to 330 and 415mg-L^{-1} respectively in glucose-alanine medium); and 400mg-L^{-1} for pyruvate and 700mg-L^{-1} for \(\alpha\)-ketoglutarate in starch-aspartate medium (compared to 300 and 930mg-L^{-1} respectively in glucose-aspartate medium). Overall the profiles were fairly similar (compare Figures 3.61 to 3.10 and 3.57 to 3.18). In this respect it was surprising that acid levels were so low in starch-nitrate cultures. The early increase in biomass was no slower in starch-nitrate medium (Figure 3.50) than glucose-nitrate medium (Figure 3.5) and in fact starch was used more rapidly (Figure 3.51 compared to Figure 3.6). The results do demonstrate the general increase in acid overproduction caused by the presence of amino acids.

Organic acids levels again increased in maltose-aspartate medium, although were lower than aspartate cultures with glucose or starch. Low acid production in maltose-containing media may be related to the relatively poor utilisation of this carbon source (Figures 3.55 and 3.65). This may reflect a restriction in the metabolism of maltose in a similar manner to that of fructose (sucrose and dextrin were also not good carbon
sources), although this is odd since starch was utilised so well. Together with reduced acid production, growth characteristics were also poor compared to glucose and starch-based media. The delayed utilisation of maltose implies that this would not be a good alternative carbon source to choose if attempting to reduce acid production by *S. lividans* TK24.

Glycerol was also slowly utilised, but more was consumed in total and acid production was correspondingly higher (Figure 3.69). Glycerol is clearly not as readily-utilised as glucose or starch by *S. lividans* TK24. Results suggest that acid production was dependent on glycerol consumption, although it cannot be said with certainty that acid production was also not dependent on aspartate. Glycerol enters glycolysis at a point above pyruvate and could feasibly contribute carbon to both pyruvate and α-ketoglutarate. A summary of maximum acid production on different carbon source is given in Section 3.7.2.

What can be concluded from Sections 3.2 and 3.4 is that all of the major carbon sources present in MEP medium (glucose, maltose and glycerol) are capable of supporting excessive organic acid production, especially when combined with amino acids. This is of course the situation encountered in MEP medium due to the presence of peptones. It was therefore no surprise that pyruvate and α-ketoglutarate were detected in cultures grown in this medium. Since it was established that amino acids influenced the levels of pyruvate and α-ketoglutarate relative to each other, and since the carbon skeletons of the vast majority of the amino acids in the peptones ultimately enter the TCA cycle (Table 4.1 and Figure 4.1), it was also not surprising that maximum α-ketoglutarate levels exceeded pyruvate levels in MEP(10 mM PO₄³⁻) medium (Figures 3.76 and 3.111). The same was true of both MEP(10 mM PO₄³⁻)-ammonium and MEP medium without phosphate supplement. In the former acid levels were reduced by around 85% due to ammonium presence (Figure 3.107); while in the latter biomass increase and α-ketoglutarate increase were delayed (Figures 3.78 and 3.80), probably due to phosphate deficiency. Acid maxima from the complex cultures was summarised in Table 3.14 (Section 3.5.3).

Several aspects of the MEP(10 mM PO₄³⁻) shake flask cultures varied from the data for pH-controlled 5L stirred-vessel fermentations using the same organism, as reported by Wrigley-Jones (1991). In the fermenter, biomass accumulation occurred earlier and without the 24h lag phase seen in the shake flasks. Both biomass and acid
accumulation ceased at 48h, while in shake flasks acid production continued until 72h and biomass increase until 96h. Acid reuse commenced before the exhaustion of glycerol in shake flasks, but this was thought by Wrigley-Jones (1991) to be the cause of acid reassimilation in stirred-vessel cultures. However, not all of the glycerol was consumed in the shake flasks cultures. Perhaps in the fermenter, where biomass reached up to 17g L⁻¹ by 48h (compared to 6g L⁻¹ by 96h in the shake flasks), glycerol was exhausted to support early rapid growth, with rapid acid reuse after 48h occurring to counter this limitation. In fermentations without pH control the pH fell as low as 2.0 and rose to 8.5-9.0 by 120h; in shake flasks the pH fell to 4.5 and rose to 5.0 by the end of each culture. These results show that the profiles differed somewhat in different vessels, although it is likely that the acids produced were the same.

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>% w/w BACTO-PEPTONE</th>
<th>% w/w MYCO-PEPTONE</th>
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<tr>
<td>Alanine*</td>
<td>6.08</td>
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</tr>
<tr>
<td>Arginine</td>
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</tr>
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<td>Aspartate</td>
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<td>Glutamate</td>
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<td>12.14</td>
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<td>Glycine*</td>
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</tr>
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</tr>
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<td>Valine</td>
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<td>4.36</td>
</tr>
</tbody>
</table>

Table 4.1: Typical breakdown of the amino acid content in the peptones present in complex media used for the shake flask cultures described in Sections 3.5 and 3.6 (from the Oxoid Manual, 5th edition, 1982). Carbon from those marked with an asterisk enter the central metabolic pathways at pyruvate (see Figure 4.1).

α-Ketoglutarate levels again exceeded pyruvate levels in media from which glycerol was absent but glucose and maltose were supplied by ME or MEB, with amino
acids either added singly or from peptone. In both MEB-glutamate and MEB-aspartate cultures the pyruvate:α-ketoglutarate ratio was approximately 1:2.5. Interestingly acid levels were generally higher in MEB-glutamate than MEB-aspartate cultures whereas levels were higher in glucose-aspartate than glucose-glutamate cultures. This may be due to the high input of glutamate from myco-peptone. In MEB-only cultures the pyruvate:α-ketoglutarate ratio was again approximately 1:2.5, but total acid production was less as would be expected. In ME-aspartate cultures the pyruvate:α-ketoglutarate ratio was around 1:2, although no peptone was present in this medium. Acid maxima were greater in this medium, which was probably due to the higher initial concentrations of glucose and maltose relative to MEB-based media. Total acid production was less in all four of these media than in MEP(10mM PO₄³⁻) medium, probably due to reduced initial carbon and nitrogen availability.

Addition of nitrate to MEB or ME caused a similar effect to that in previous glucose-(amino acid)-nitrate media where maximum pyruvate levels greatly exceeded total α-ketoglutarate levels. It can be speculated that in maltose-nitrate medium pyruvate production exceeded α-ketoglutarate production to the extent that α-ketoglutarate was not detected (pyruvate production was also very low). In a selection of glucose and nitrate-containing media, pyruvate:α-ketoglutarate ratios were around 3:1 (glucose-nitrate), 5:1 (glucose-aspartate-nitrate) and 4.5:1 (glucose-alanine-nitrate). In MEB-nitrate cultures the ratio was about 6.5:1 and in ME-nitrate 4:1. Accounting for the extra sugars and amino acids in the complex media, MEB-nitrate cultures obviously resembled glucose-amino acid-nitrate cultures, while ME-nitrate resembled the glucose-nitrate pattern.

As expected no acids were detected in peptone-nitrate cultures, which lacked a readily-used carbon source such as glucose or glycerol. It is unfortunate that cultures in a medium containing, for example, 5g·L⁻¹ glucose and 10g·L⁻¹ peptone were not conducted, but it can be assumed that organic acids would have been produced.
4.2.2 A Theoretical Evaluation of Pyruvate and α-Ketoglutarate Over-Production

A number of theories explaining organic acid excretion have been suggested by other authors. Ahmed et al. (1984) suggested that pyruvate and α-ketoglutarate excretion by *S. venezuelae* may be due to the suppression of dehydrogenase enzymes (PDH and α-KDH) by glucose. PDH and α-KDH activities were low or absent in high-acid-producing cultures. Surowitz & Pfister (1985) speculated that pyruvate accumulation by *S. alboniger* was due to increased activity of glycolytic pathway enzymes relative to TCA cycle enzymes. These authors observed increases in the activities of certain glycolytic enzymes (*e.g.* phosphofructokinase and pyruvate kinase) but not a decrease in PDH activity during acidifying cultures. This strain only excreted pyruvate. Dekleva & Strohl (1987) also detected both pyruvate and α-ketoglutarate in the medium of *S. peucetius* cultures but dehydrogenase enzyme levels did not vary, leading these workers to suggest that acid excretion was related to the transport and metabolism of specific acid-inducing sugars such as glucose.

Although time did not permit the assaying of enzyme levels during this project, the nature of the cause of acid excretion may be speculated upon. Firstly, it is possible that acid excretion occurred simply due to an overloading of the central metabolic pathways during excessively rapid carbon source uptake, as suggested for acidifying *E. coli* cultures (El-Mansi & Holms, 1989; Section 1.2.2). During this project it was found that organic acid excretion generally occurred at its fastest rate during the period in which the primary carbon source was consumed the most rapidly. This can be seen in the profiles from cultures in a number of different media including, at the simplest level, glucose-nitrate (Figure 3.6). Acid concentrations increased gradually between 30 and 48h during the gradual initial uptake of glucose when the concentration fell from 5.87g.L⁻¹ at 0h to 5.47g.L⁻¹ at 48h. The rapid utilisation of glucose began at 48h until exhaustion at 78h. In this period the acid concentrations increased rapidly until both peaked at 78h. It has been noted that of the glucose carbon consumed over 78h, up to 11% was excreted as organic acids, not accounting for any reused when the glucose fell to low levels at around 72h. A similar pattern of rapid increase in acid concentrations during the rapid utilisation of glucose could be seen during glucose-amino acid cultures (Figures 3.10, 3.14, 3.18, 3.22 and 2.36). The occurrence of the α-ketoglutarate peak after carbon source exhaustion in these media may reflect the conversion of carbon from
both sources to acids in these media. A similar pattern can be seen in glucose-amino acid-nitrate cultures (Figures 3.36 and 3.40).

The same patterns were seen with alternative carbon sources (Figures 3.57, 3.61, 3.65 and 3.69). Delays in acid increase in starch-containing media may reflect the number of steps required to generate glucose from this polymer; Figures 3.59 and 3.63 do show a rapid increase in organic acid concentrations occurring during or immediately after rapid starch uptake.

If the utilisation of the readily-measured principal carbon sources from complex media is studied then the same trend is seen. An example is MEP(10mM PO_4^{3-}) medium (Figures 3.75 showing the sequential rapid use of glucose and maltose from 30 to 54h and then glycerol from 54 to 120h; and 3.76 showing rapid organic acid accumulation between 30 and 72h); others are described in Section 3.5.2.

An equivalent trend is apparent in the acidifying cultures of *S. coelicolor* reported by Hobbs *et al.* (1992). In glucose-alanine defined medium, glucose was utilised most rapidly from 48 to 66h, coincident with a rapid increase in the concentrations of pyruvate and α-ketoglutarate to peaks at around 66h and 70h respectively.

El-Mansi & Holms (1989) suggested that when input carbon exceeded the capacity of the central metabolic pathways and the requirements for biosynthesis and energy generation, the overload of excess carbon could be disposed of by the excretion of low molecular weight metabolites (acetate for *E. coli*), *i.e.* a metabolic imbalance was occurring. Subsequently these and other workers (*e.g.* Konstantinov *et al*., 1990; Turner *et al*., 1994) demonstrated that restriction of the glucose supply by feeding reduced acid excretion. This would imply that *E. coli* has no regulatory control over sugar uptake when presented with an excess and that taking up more than is needed results in excretion of organic acids (also noted by Turner *et al*., 1994). A similar scenario can be envisioned for *S. lividans* such that the uptake of excess carbon source not needed for essential cellular functions is balanced by the excretion of organic acid intermediates. Such a metabolic imbalance was alluded to by Wrigley-Jones *et al.* (1993). The same could be implied by the results of Surowitz & Pfister (1985) where increased glycolytic activity (perhaps due to excessive glucose uptake by *S. alboniger*) apparently caused pyruvate excretion.

It should be noted that glucose was utilised equally rapidly from some non-acid-producing *S. lividans* cultures (*e.g.* glucose-aspartate-ammonium, Figure 3.30), although
biomass was improved slightly (Figure 3.29) and the lack of acids may be related specifically to the presence of ammonium (discussed later in this section). It would be interesting to investigate whether the feeding of carbon sources (in fed-batch fermentations for example) could reduce acid production by *S. lividans* TK24. Potential future work is discussed in Section 4.4.

Alternatively, acid excretion by *S. lividans* may be caused by glucose repression of the dehydrogenase enzyme complexes PDH and α-KDH which degrade pyruvate and α-ketoglutarate respectively. Guest (1992) notes that most of the TCA cycle enzymes in *E. coli* are repressed by glucose, including both PDH and α-KDH. The observation that PDH and α-KDH activities are deficient in high-acid-producing *S. venezuelae* cultures grown in glucose-based defined medium led Ahmed *et al.* (1984) to suggest that pyruvate and α-ketoglutarate excretion was due to glucose repression of these enzymes. Unfortunately these authors did not investigate alternative carbon sources to glucose.

It is feasible that the excretion of pyruvate and α-ketoglutarate by *S. lividans* might be due to glucose repression. Maltose and starch, both polymers of glucose, also supported acid production and both can cause carbon repression (Section 1.3.2). Since glycerol is also capable of causing carbon repression, it would be possible for glycerol to also support acid production. Repressive and non-repressive carbon sources vary greatly between streptomycete strains and pathways (refer to Section 1.3.2; also Martin & Demain, 1980 and Demain, 1992), as does the dependence of acid production on different carbon sources (see Section 4.1). Pyruvate and α-ketoglutarate excretion by *S. peucetius* was observed by Dekleva & Strohl (1987) in glucose or fructose-based defined media, but not in those containing maltose or starch. In contrast *S. lividans* produced acids in glucose, maltose and starch-based defined media but not in fructose media. Dekleva & Strohl (1987) observed neither a decrease in PDH and α-KDH activity (like Ahmed *et al.*, 1984) nor any increase in the enzymes preceding pyruvate and α-ketoglutarate (like Surowitz & Pfister, 1985). Moreover, since glucose polymers did not cause acid production, the authors concluded that organic acid production was related to the specific metabolism (*e.g.* uptake and phosphate activation) of carbon sources such as glucose, rather than to glucose repression. Of course, it has been suggested that carbon repression in some *Streptomyces* strains involves the enzyme glucose kinase (GLK) which phosphorylates glucose directly after uptake (Hodgson, 1982; Seno & Chater, 1983; see Section 1.3.2). The absence of GLK in *S. coelicolor*
affected glucose repression of a number of genes (Angell et al., 1992; Servin-Gonzalez et al., 1994). Conclusive proof of the involvement of carbon source repression in organic acid overproduction by S. lividans would require the study of a number of glycolytic and TCA cycle enzymes, including PDH and α-KDH. Potential future work is discussed in Section 4.4.

What is commonly perceived as nitrogen regulation in streptomycetes (Section 1.3.3) does not seem to be applicable to the control of organic acid overproduction in S. lividans TK24. It should be noted that the theory of Ahmed et al. (1984) for S. venezuelae (discussed in Section 4.1) did resemble nitrogen regulation in that acid production only occurred under nitrogen-limitation. During this project, S. lividans TK24 excreted organic acids during growth on both rapidly-utilised nitrogen sources (most amino acids) and on poorly-used nitrogen sources (nitrate, complex amino acids) but not on another poorly-used type (ammonium). In other words, acid excretion occurred during both nitrogen availability and limitation and there was no general trend. Other authors have also reported inconsistent trends regarding nitrogen regulation (e.g. Bascaran et al., 1989a and 1989b; also Brana et al., 1985 and 1986 and Shapiro & Vining, 1983 and 1984; otherwise refer to Section 1.3.3). In addition, Fang & Demain (1995) have shown that nutrient regulation is not absolute and may be affected by environmental conditions such as restricted aeration.

Liras et al. (1990) include organic acids amongst other secondary metabolites (pigments and antibiotics) whose production is suppressed during rapid growth on a preferred nitrogen source. Following the indications discussed in Section 4.1, if this were applicable to S. lividans then organic acid excretion would be expected only after the exhaustion of preferred amino acids and not during their use; and acid excretion would occur during growth on both nitrate and ammonium, which are less readily-utilised by S. lividans. It can be presumed that specific ammonium repression of glycolytic and TCA cycle enzymes is also not involved, since if ammonium repressed these enzymes (perhaps in the way that glucose, another classically repressive substrate, may do) then organic acid excretion would be expected in media containing ammonium. In fact quite the opposite seems to apply.

Instead, if it is assumed that PDH and α-KDH are most likely to be rate-limiting and to cause a build-up of pyruvate and α-ketoglutarate during normal aerobic metabolism, then it might be that the correct action or formation of PDH and α-KDH (or
subunits of these enzyme complexes) have some subtle and essential requirement for ammonium. In this case it would be understandable that no organic acids were excreted during growth in the presence of excess ammonium. Since PDH and α-KDH are enzyme complexes, it would be complicated to study any differences in activity or formation of specific parts of the enzymes. A simple potential experiment would be to study cell extracts containing the enzymes. In this case, an assay mix containing enzyme extracts and substrates could be assayed in order to obtain a measure of the 'normal' rate of activity. Subsequently, a portion of ammonium could be added to identical assay mixtures to determine if exogenous ammonium did affect the enzymes, or whether the cause of pyruvate and α-ketoglutarate build-up lies elsewhere in metabolism. Further potential experiments are considered in Section 4.4.

If ammonium does affect PDH and/or α-KDH, then in glucose-nitrate medium, which would contain absolutely no ammonium ions, PDH and α-KDH may be rate-limiting so pyruvate and α-ketoglutarate build up and are excreted. Since the PDH blockage is encountered first as carbon flows from glucose through glycolysis and the TCA cycle, then pyruvate builds up quickly. What carbon gets through to the TCA cycle is then blocked at α-KDH and α-ketoglutarate builds up and is excreted. The relationship of amino acids and nitrate in a mixed nitrogen source medium, however, still remains unclear. In media with amino acids but no nitrate, a certain amount of ammonium will be generated from the degradation of the amino acids, which may act at a common control point to ammonium supplied in the medium. This ammonium might ease the blockage at PDH, thus reducing pyruvate levels. Although the blockage at α-KDH may also have been eased, a combination of increased carbon flow and the remaining blockage at α-KDH would conspire to increase the build-up of α-ketoglutarate. Unfortunately this hypothesis may require that ammonium is preferred to amino acids or nitrate. This is apparently negated by the fact that ammonium salts were never exhausted from the glucose-based media to which they were added (while amino acids tended to be exhausted rapidly) and in turn no acids were detected in these media. It may be that acids would have been produced had ammonium been exhausted early during each culture. Unsuccessful attempts to induce ammonium exhaustion in some S. lividans cultures were discussed in Section 4.1.

A recent article by Obanye et al. (1996) reported that carbon flux through the central metabolic pathways of S. coelicolor varied during the phases of growth, which was associated with antibiotic production. Pyruvate and α-ketoglutarate excretion were
thought to be due to metabolic and transport changes during growth. Pyruvate increase and decrease appeared to be related to variations in the ratios of glycolysis and the TCA cycle, and subsequent alterations in the flux through PDH involving the NADH/NAD⁺ ratio. It is possible that similar events could occur during the growth of \( S. \text{lividans} \), which requires further study.

The PDH and α-KDH complexes share a number of common features. These analogous enzymes are both large complexes which oxidatively degrade their substrates, reduce NAD⁺ to NADH and couple the product of decarboxylation to CoA (Saier, 1987; Stryer, 1995). The reactions are shown in Equations 4.1 and 4.2.

\[
\text{pyruvate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{PDH}} \text{acetyl CoA} + \text{CO}_2 + \text{NADH} \\
\text{α-ketoglutarate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\alpha-\text{KDH}} \text{succinyl CoA} + \text{CO}_2 + \text{NADH}
\]

(Equation 4.1)

(Equation 4.2)

Each complex consists of 3 enzymes, a specific PDH or α-KDH component (\( E_1 \) or \( E'_1 \)), a dihydrolipoyl transacetylase in PDH (\( E_2 \)) or a dihydrolipoyl transsuccinylase in α-KDH (\( E'_2 \)) and a dihydrolipoyl dehydrogenase (\( E_3 \) or \( E'_3 \)). It is known that the \( E_3 \) subunit is identical in both complexes (Saier, 1987; Stryer, 1995) and in \( E. \text{coli} \), this subunit is encoded by a single gene (Guest, 1992). Other commonly shared features include the catalytic co-factors thiamine pyrophosphate, lipoamide and FAD and the stoichiometric co-factors NAD⁺ and CoA. These provide a number of points at which the enzymes could both be affected by carbon or nitrogen sources, thus causing overproduction of both pyruvate and α-ketoglutarate.

Both enzymes are important control points in the central metabolic pathways and are negatively regulated by excess product and NADH. PDH is also negatively regulated by excess ATP. α-KDH is possibly regulated by ATP (Zubay, 1993). NADH is generated during the rapid flux of carbon source through glycolysis (notably at a point below the entry of both glucose and glycerol; see Figure 1.1) and during the TCA cycle. ATP is also generated during glycolysis. A certain amount of each is generated before both PDH and α-KDH. During excessive carbon source uptake it is possible that this regulation may also suppress enzyme activity and cause blockages at PDH and α-KDH,
resulting in build-up and subsequent excretion of pyruvate and α-ketoglutarate. PDH may also be regulated by reversible phosphorylation of the E₁ component, although strictly excess pyruvate should inhibit the kinase and reactivate the PDH (Saier, 1987; Stryer, 1995). This would make it similar to mammalian PDH, which is possible since streptomyces and mammalian α-amylases have recently been found to share similar features (Long et al., 1987; Virolle et al., 1988). The specific regulation of PDH and α-KDH in *S. lividans* TK24 would also be interesting to study.
4.3 Is Morphology Related to Organic Acid Excretion?

It is possible that excessive organic acid excretion by \textit{S. lividans} TK24 was related to the morphology of the biomass, particularly the formation of hyphal pellets, and subsequent oxygen and nutrient limitation. The defined medium used for this project (SMM, Section 2.1.3) was initially optimised for a reasonable biomass yield by this strain and the complex medium (MEP) was known to support good growth (Wrigley-Jones, 1991). Despite the use of stainless steel springs in shake flasks, which have been reported to aid dispersed growth by \textit{S. coelicolor} (Hodgson, 1982), \textit{S. lividans} TK24 persistently exhibited a tendency towards pelleted morphology. Magnolo \textit{et al.} (1991) also reported that \textit{S. lividans} formed pellets, even when grown in complex medium (which is generally regarded to favour dispersed growth; Whitaker, 1992) in shake flasks containing steel springs. Since this has been encountered by other authors (\textit{e.g.} Hobbs \textit{et al.}, 1989), this does not seem to be an uncommon problem with \textit{S. lividans}. Of the other preventative methods suggested (Section 1.4.2), polymers (Hobbs \textit{et al.}, 1989) were found to interfere with HPLC analysis and equipment; while glass beads (Doull & Vining, 1989) had no better effect than springs and were more difficult to handle. The use of springs was found to increase the tendency towards dispersed growth and reduced pellet size. In a series of early defined and complex medium shake flasks in plain and baffled flasks using varying inoculum concentration but standard incubation conditions (data not shown), \textit{S. lividans} TK24 was found to form very large bead-like pellets which were around 2mm across, smooth and shiny. No dispersed mycelium was observed at all. Subsequent cultures with springs were found to encourage an approximately 50:50 mixture by weight of pellets and dispersed mycelia in most defined and semi-complex media; or the formation of predominantly dispersed mycelia with few pellets in full MEP complex medium. The details are discussed below.

Pelleting affects the growth and metabolism of \textit{Streptomyces} cultures, since the internal regions of the pellets may become deprived of nutrients (Bader, 1986) and oxygen (Bushell, 1988). It has already been noted that oxygen deficiency in pellets may encourage the excretion of TCA cycle-associated organic acids by filamentous fungi (Bushell, 1988). These are not just acids such as lactate which might be expected under anaerobiosis, but other types such as citrate, which indicates specific metabolic imbalances or blockages in pelleted mycelia. The same may be applicable to \textit{Streptomyces}. Subsequently, low pH caused by undesirable acid excretion in

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*Streptomyces* cultures may encourage further pellet formation, as the pH shifts from the optimum for growth (Prosser & Tough, 1991). Nutrient limitation in pellets may cause the onset of secondary metabolic activity (Doull & Vining, 1990a) and it has been noted by some authors (*e.g.* Liras, Asturias & Martin, 1990) that excreted organic acids may be regarded as secondary products, presumably in that they are not essential for growth following the definition of Martin & Demain (1980). A review of the causes and effects of pelleting is given in Section 1.4.2.

Reviewing first the defined medium shake flasks presented in Sections 3.2.2.1 to 3.2.2.3, the absence of pelleting can be correlated with the absence of organic acid excretion. Glucose-ammonium cultures contained mainly dispersed mycelia with a few tiny pellets, which was unusual for defined media. No organic acids were detected, although this may be due to the presence of ammonium ions as discussed in Section 4.2. Biomass was observed to be in the form of a mixture of small pellets and dispersed mycelia in all of the following cultures, all of which excreted organic acids: glucose-nitrate, glucose-alanine, glucose-glycine, glucose-aspartate, glucose-glutamate, glucose-lysine, glucose-aspartate-nitrate and glucose-alanine-nitrate. Growth was dispersed in glucose-aspartate-ammonium cultures in which no acids were detected; but heavy pelleting was observed in glucose-alanine-ammonium cultures in which a small amount of pyruvate was excreted. It may be that the preventative effect of ammonium was 'over-ridden' by the deleterious effects caused by pelleting. Pellet formation was also observed in shake flasks containing radiolabelled substrates (Section 3.3).

Most of the cultures described in Section 3.2.2.4 (amino acids with or without extra nitrogen source, but without glucose) also exhibited pelleted morphology. If pelleting did encourage acid excretion then the lack of acids in these cultures may have been due to the lack of a rapidly-utilised carbon source in the form of glucose. Pelleting was conspicuously absent from alanine-ammonium cultures. It is interesting that these cultures also achieved the highest biomass levels recorded for defined media. This suggests that pelleting also has deleterious effects on growth, although pelleting may itself be a consequence of poor growth. In all cultures conducted during this project, when pellets did form they were visible from the first sample at 24h and throughout the course of each culture.
No pellets were observed during cultures grown in defined media with an alternative carbon source and nitrate (Section 3.4.2.1). Although organic acids were detected in the medium of starch-nitrate and maltose-nitrate cultures, their concentrations were lower than might have been expected from (i) the similarity of the metabolism of these carbon sources to glucose; and (ii) the higher acid production recorded in cultures with starch or maltose and an amino acid, in which pelleting did occur (Sections 3.4.2.2 and 3.4.2.3). The enhanced biomass yields in starch-nitrate (2.8g·L⁻¹) and maltose-nitrate (2.7g·L⁻¹) cultures compared to glucose-nitrate (2.1g·L⁻¹) may be explained by the dispersed growth form and/or reduced acid production. The biomass yield was only fractionally enhanced in starch-amino acid cultures (2.9-3.0g·L⁻¹) considering the extra input from the amino acids, and was actually less in maltose-aspartate medium (2.3g·L⁻¹).

Glycerol-aspartate cultures (Section 3.4.2.3) exhibiting pelleted growth also excreted large amounts of organic acids. By comparison, the lack of any acid in the extracellular medium of glycerol-nitrate cultures (Section 3.4.2.1) is odd and might be explained by the lack of pellets in these cultures.

The failure of fructose-grown cultures to produce acids whether pelleted or not could be due to the poor utilisation of this carbon source by S. lividans TK24. In this case, the carbon-limitation suffered might resemble media lacking a sugar carbon source (Section 3.2.2.4) from which acidification was absent.

Shake flask cultures in complex MEP medium exhibited a slight degree of pelleting. In full MEP medium with or without extra phosphate and ammonium supplements (Sections 3.5.2.1 and 3.5.2.4), growth was predominantly in a dispersed form. Pelleting was not as pronounced as defined media, although a significant number of small pellets were visible. The biomass yield was also good in these media (excepting MEP which was phosphate-limited). Organic acid excretion was high but lower than might have been expected from the higher carbon input. This might of course also be connected to the higher biomass yield. From the cultures described in Section 3.6.2, it was known that of the consumed carbon, around 6% was excreted as organic acids, compared to an average of over 11% in defined media. It is possible that the conversion of carbon to acids was reduced (and that to biomass enhanced) by the nature of the complex medium (as seen in E. coli cultures by Koh et al., 1992), by phosphate
buffering effects (suggested by Wrigley-Jones, 1991), or because pelleting was less compared to defined media.

In semi-complex media (Sections 3.5.2.2 and 3.5.2.3) pelleted growth was more common, giving an approximately 50:50 mixture of pellets and dispersed mycelia. This could be correlated both to the relatively increased excreted acid concentrations and to the decreased biomass yields. Peptone-nitrate cultures, with little pelleting and no acids, can be directly compared to defined media lacking glucose (Section 3.2.2.4).

From these observations it might be suggested that pelleted growth was related to copious organic acids excretion by \textit{S. lividans} TK24. Alternatively pelleting might have increased acid production by aggravating nutrient and/or oxygen-limited conditions in a large proportion of the mycelium in each culture. In stirred vessel fermentations (Section 3.7.2) pelleted growth was also observed together with acid production (Figure 3.119). Levels were lower than might have been expected compared to the equivalent shake flask cultures. At 72h, the pyruvate and \(\alpha\)-ketoglutarate concentrations were 250 and 674mg-L\(^{-1}\) respectively, compared to the shake flask levels at 72h of 300 and 797mg-L\(^{-1}\) respectively (Figure 3.18). Biomass was also lower in the fermenter, which may have been related to the lower acid production.

In conclusion, it is possible that pelleted growth might be related to acid production. This would also be an interesting aspect of \textit{S. lividans} TK24 growth to study further. It is also possible that pelleting was the cause of generally poor growth characteristics, for example overall linear rather than exponential growth was common in pelleted cultures (confirmed by Prosser & Tough, 1991). In pellets, interior mycelia may be starved of nutrients and oxygen and cease to grow, while exterior mycelia continue to grow exponentially (Bader, 1986; Bushell, 1988).
4.4 Prevention of Organic Acid Excretion and Future Directions

From the results presented in Section 3 and discussed so far in Section 4 it is possible to propose ways in which acid excretion by *S. lividans* TK24 might be reduced or prevented. It is also possible to consider the future directions of this area of study. As such, the information gained during this project will potentially be of use in the rational improvement in yields of recombinant products from *S. lividans*, by minimising the flow of precursors to undesirable organic acid by-products.

In a batch fermentation situation, it can be assumed that the conversion of a significant portion of the carbon consumed by a culture to by-products such as organic acids represents a wastage. In defined medium shake flask cultures of *S. lividans* the maximum acid concentrations recorded indicated that an average of 11.2% of the consumed carbon was excreted as acids, decreasing to 6% in complex medium. It was observed that pyruvate was rapidly reused from many cultures. Some reassimilated pyruvate carbon may then have been excreted as α-ketoglutarate, therefore the above percentages may be generous. While pyruvate was usually completely reused this was rarely the case with α-ketoglutarate. The α-ketoglutarate remaining unused in the culture medium represented a wastage of carbon. For example in glucose-alanine medium (Figure 3.10) at 72h just before entry into the stationary phase and while carbon was still required by the culture (Figure 3.9), the carbon present as α-ketoglutarate represented 6.3% of the carbon consumed by that point in the culture. By 120h, α-ketoglutarate in the medium still represented around 3.7% of the total consumed carbon (Figure 3.12).

In addition, α-ketoglutarate is specifically required for anabolic pathways as the major metabolic precursor for the formation of a number of amino acids, for example glutamate, glutamine, arginine and proline (Saier, 1987). These provide building blocks for the synthesis of proteins and biomass (Stryer, 1995). Hence, any restriction in the availability of α-ketoglutarate (due to its excretion) and in carbon reaching the rest of the TCA cycle and other amino acid precursors (*e.g.* oxaloacetate, precursor of aspartate, asparagine, methionine, threonine, isoleucine and lysine) might be deleterious to the cells. Of course, it is likely that some of the α-ketoglutarate which builds up in the cells would be used to synthesise amino acids by routes (*e.g.* GDH, GS, GOGAT) other than those which contain the blockage.
It is fairly easy to control pH in stirred vessel fermentations. In either shake flasks or uncontrolled fermentations it is possible that pH fluctuations might affect both growth and product synthesis by a streptomycete culture (refer to Sections 1.1.1.2 and 1.4). It has been suggested that the fall and characteristic later rise in pH might affect a secreted recombinant product (Wrigley-Jones *et al.*, 1993). An enzyme might be denatured or otherwise inactivated in these conditions. Lee & Lee (1994) found that both production and activity levels of β-lactamase cloned into *S. lividans* was optimum at pH 7.0 and sharply decreased at pH 6.0 or 8.5.

Previously pH fluctuations in *Streptomyces* cultures have been prevented or reduced by the use of buffers such as MOPS (Aharonowitz & Demain, 1977; also Chatterjee & Vining, 1982) although notably MOPS was not used during the subsequent organic acid studies of Ahmed *et al.*, 1984). In these reports it was not specifically stated if MOPS prevented acid excretion or simply prevented the pH from changing. Shapiro & Vining (1983) found that MOPS interfered with *S. venezuelae* fermentation characteristics and analysis, and switched to using pH-controlled fermenters. Zhang *et al.* (1996) were unable to prevent the pH falling in cultures of *S. griseofuscus* in defined glucose-ammonium medium containing MOPS. These authors resorted to the use of a calcium carbonate supplement to maintain a stable pH. Therefore the use of MOPS buffer may not be suitable for all *Streptomyces* strains.

During this project ammonium ions were found to prevent acid excretion by *S. lividans* TK24 in defined media. Ammonium supplements in complex media reduced extracellular acid levels substantially. From the literature it appears that this effect is not consistent for all streptomycetes, and the preference for ammonium and its relationship to acid production appears to vary between strains (*e.g.* Ahmed *et al.*, 1984). In addition ammonium ions are known to affect secondary metabolic pathways (Section 1.3.3). Therefore care should be taken if attempting to use ammonium to suppress acid production and increase the yield of recombinant products produced by *S. lividans* TK24, especially antibiotics from cloned pathways. Ammonium also appears to interact with other nitrogen sources and prevent the best possible growth (*e.g.* in glucose-alanine-ammonium medium).

An alternative choice for a medium formulation discouraging acid overproduction might be the use of extra phosphate. Aharonowitz & Demain (1977) reported that a high concentration of potassium phosphates stabilised pH fluctuations in cultures of *S. clavuligerus*. Wrigley-Jones (1991) suggested that the extra phosphate present in
shake flask cultures of *S. lividans* TK24 in MEP(10mM PO$_4^{3-}$) medium provided a better buffering capacity, and allowed better growth characteristics and yield than unsupplemented MEP medium. However, this author also reported that an equal amount of alkali was needed to counteract acid production in MEP(10mM PO$_4^{3-}$) and MEP media in a 5L working volume fermentation (125mL for the TK24 host or 115 and 140mL respectively for a plasmid-containing strain). The biomass yield was about the same in both cultures at 10 to 12g·L$^{-1}$ DCW. During this project it has been found that in shake flasks the total acid concentration was actually higher in MEP(10mM PO$_4^{3-}$) than MEP medium (refer to Section 3.5.2.1). It is therefore likely that both acid production and the biomass yield were less in MEP medium due to phosphate-limitation. Hobbs *et al.* (1992) used high phosphate concentrations to direct antibiotic synthesis by *S. coelicolor* but excretion of pyruvate and α-ketoglutarate was still observed.

It is known that excess phosphate also affects secondary metabolism, hence the reason that Aharonowitz & Demain (1977) switched from phosphate to MOPS buffer to control pH fluctuation in cultures of *S. clavuligerus*. Phosphate limitation was clearly found to cause intense pigmentation in *S. lividans* TK24 cultures in MEP media (Figure 4.2). No pigmentation occurred in MEP(10mM PO$_4^{3-}$) medium. Pigmentation was thought to be due to the production of the antibiotics and is summarised later in this section. Hence, the use of excess phosphate may not actually reduce acid excretion and could interfere with certain recombinant products such as antibiotics, which also requires further study.

*Figure 4.2: Samples from cultures grown in (a) MEP(10mM PO$_4^{3-}$) medium and (b) MEP medium showing the presence or absence of intense pigmentation.*
It has been reported that carbon source feeding can reduce bacterial acid by-products (Payne et al., 1990; Konstantinov et al., 1990). Wrigley-Jones (1991) also reported that acid production was reduced during an induced stirred vessel fermentation. The author suggested that this was due to the crude fed-batch nature of such a fermentation and that true fed-batch conditions might prevent acid excretion and improve biomass and product yield. It was unfortunate that equipment problems (Section 3.8) did not permit the feeding experiments hoped for. However, it would not be difficult to measure acid production and control the feed rate in fed-batch fermentations in a manner similar to Turner et al. (1994). This would be especially useful if the overload theory presented in Section 4.2.2 were true.

Finally it has been suggested that acid production by *E. coli* may be reduced in complex medium (Koh et al., 1992; Han et al., 1992). The wasteful excretion of consumed carbon as organic acids did appear to be less in complex media (Sections 3.5 and 3.6) than in defined media (Sections 3.2 and 3.4). Therefore, it may be prudent to combine the use of complex media with the other preventative methods reviewed here in order to minimise organic acid excretion by *S. lividans* TK24.

For future study in this area it would be interesting to investigate the effect of carbon source feeding on acid production; also the potential role of pelleted morphology in aggravating acid levels. Initially, a more comprehensive series of stirred vessel fermentations might be undertaken to investigate acid excretion by *S. lividans* TK24 when grown under more controlled conditions. Aspects of interest would include the influence of pelleting, also perhaps a more in-depth study of pelleting in relation to varied medium composition, inoculum size and environmental parameters (stirrer speed/aeration). Another factor affecting acid excretion may be oxygen transfer. It may be worthwhile to study the relationship of DOT and acid excretion. It is already known from the work of Wrigley-Jones (1991) that acid excretion by *S. lividans* TK24 was as high in cultures in MEP(10mM P<sub>4</sub>O<sub>4</sub><sup>3-</sup>) medium under restricted aeration (0.48 meq acid) compared to equivalent cultures with good aeration (0.55 meq). The principal difference was that acids were not reused from oxygen-deficient cultures. It was suggested by Wrigley-Jones et al. (1993) that in anaerobic conditions the organism would not be able to reuse organic acids due to the lack of oxygen as a terminal electron receptor. Both oxygen and mass transfer effects/restrictions in pellets might also be considered in light of acid excretion (refer to Section 4.3). Finally, the physical aspects of pH control on
acid excretion would be useful to investigate. Principally, are acids still excreted when
the medium pH is actively controlled? In which case, does low pH actually affect uptake
mechanisms by the cells? If acids are still excreted, are levels the same, higher or lower
than in uncontrolled fermentations? These are important when considering the potential
effects of acid excretion on a recombinant product in terms of carbon wastage.

It would be very useful to investigate the role of glycolytic and TCA cycle
enzymes in organic acid over-production by \textit{S. lividans} TK24. The first aspect to study
would be the activities of a selection of enzymes including PDH and \(\alpha\)-KDH. In
particular, do the activities of any enzymes decrease (\textit{e.g.} Ahmed \textit{et al.}, 1984) or
increase (\textit{e.g.} Surowitz & Pfister, 1985) in acid-producing relative to non-acid-
producing cultures? Do the activities of any enzymes change with different carbon and
nitrogen sources, and is acid excretion related to the rate or method of nutrient uptake?
Is any enzyme limiting in nitrate/amino acid media compared to ammonium media (an
experiment to investigate the effect of ammonium on PDH and \(\alpha\)-KDH was suggested in
Section 4.2.2)? Once the enzymes at the root of acid overproduction had been identified
then future investigation in this area might involve in depth analysis of enzyme
regulation mechanisms. Potentially, regulation could occur at the transcriptional level.
When the enzymes had been identified (for example, if it is assumed that PDH and/or \(\alpha\)-
KDH blockage cause acid excretion) then the next step would be to locate the genes for
the enzyme components (detailed in Section 4.2.2). In \textit{E. coli}, the genes encoding the
E'\(_1\) and E'\(_2\) components of the \(\alpha\)-KDH complex are held in one cluster of genes which
also contains the genes for citrate synthase, succinate dehydrogenase and succinyl CoA
synthetase. The genes for the E\(_1\) and E\(_2\) components of PDH are located in a second
cluster which also contains the gene encoding the common E\(_2\)/E'\(_3\) component (Guest,
1992). Interestingly, some of these other enzymes are major metabolic control points.
SDH is also repressed by glucose or anaerobiosis in \textit{E. coli} (like PDH and \(\alpha\)-KDH; 
Guest, 1992); CS is regulated allosterically (Stryer, 1995). Having identified the genes,
transcript levels could be studied to ascertain if levels varied in certain acidifying
cultures relative to other acidifying cultures and non-acidifying cultures. Experiments
monitoring transcript levels are common and are reported by, \textit{e.g.}, Hobbs \textit{et al.}, 1992.

Should the control of the enzymes at fault not be at the transcriptional level, then
further investigation might involve study of the translational machinery (\textit{e.g.} availability
of correct tRNA units or amino acids at the time of acid excretion) or of any inhibition
of enzyme activities. Study of enzyme activities would probably be required to identify
the enzymes causing pyruvate and α-ketoglutarate build-up. Subsequently, activities of various enzymes could be compared in cultures exhibiting different degrees of acidification, for example glucose-ammonium (no acids), glucose-nitrate (high pyruvate, low α-ketoglutarate) and glucose-aspartate (lower pyruvate, higher α-ketoglutarate). Similar experiments have been reported by other authors. Ahmed et al. (1984) assayed PDH and α-KDH activities in cell extracts prepared by sonication, using sodium pyruvate or potassium α-ketoglutarate as substrates, and measuring the change in absorbance at 365nm. Other methods are reported by Dekleva & Strohl (1987) and Surowitz & Pfister (1985) for enzymes including PDH, α-KDH, pyruvate kinase and citrate synthase.

Other factors which might affect metabolic enzymes include environmental factors such as oxygen levels in the medium and the variation of this with agitation/stirrer speed (which also affects pelleting); pH (again related to pelleting); and medium composition, particularly the levels of salts. Once methods had been established to monitor levels of enzyme transcripts or activities, it would then be worthwhile to establish the effects of these external parameters on the internal environment. Finally, acid excretion may be affected by other intracellular factors. Preliminary investigations indicate that the 24h lag in growth at the start of each S. lividans TK24 culture may be due to low levels of intracellular effectors such as ATP. Further study and metabolic modelling might reveal correlations between levels of intracellular factors and acid overproduction.

If acid excretion is caused by variation in enzyme activity or by overloading of the central metabolic pathways with carbon, then it may be possible to use genetic manipulation to alleviate these problems. Chater (1990) noted that it would be possible to enhance precursor flow to desired pathways by inactivating undesirable competing pathways assuming they are dispensable. Since pyruvate and α-ketoglutarate are essential to cellular function then it would not be a good idea to block their production altogether. Instead if enzymes are overactive then it may be possible to restrict or divert carbon flow to these intermediates at a higher level. Takebe et al. (1991) reported that high bialaphos-producing strains of S. hygroscopicus had a metabolism where the flow of carbon from pyruvate and acetyl CoA (precursors of bialaphos) to the TCA cycle was suppressed. Although not caused by metabolic engineering, this example does show the
potential for redirection of carbon flow in a recombinant to a desired product and away from organic acids.

Alternatively if enzymes are rate-limiting and cause pyruvate and α-ketoglutarate to build-up then it may be possible to clone extra copies of the genes encoding these enzymes into the host. Bailey (1991) reviewed how the expression of the Zymomonas mobilis enzymes pyruvate decarboxylase and alcohol dehydrogenase in E. coli decreased acid excretion and increased biomass levels. The enzymes redirected the flux from pyruvate and rechannelled carbon to ethanol which was less inhibitory to growth when excreted. Mutants insensitive to the control of limiting enzymes could also be developed once the enzymes had been identified.

Finally, it would be interesting to firmly establish that organic acid excretion by S. lividans TK24 does affect recombinant products. Initially the native pigment antibiotic products observed in S. lividans TK24 cultures could be used. Most defined cultures became pigmented. Biomass material turned orange-red around 48h, which was thought to be caused by undecylprodigiosin production during rapid growth (Section 1.1.2.2). The medium later became coloured, presumably due to synthesis of the diffusible pH indicator actinorhodin (red in acidic cultures, blue or purple in neutral or alkaline cultures). The medium colour changed as excreted acids were reused. Preliminary assays following the methods of Horinouchi & Beppu (1984) indicated that this pigment was indeed actinorhodin.

Subsequent investigation of acid effects might involve α-amylase (e.g. Bahri & Ward, 1990), or others from the plethora of recombinant products for which S. lividans TK24 has proved itself to be a suitable host.
REFERENCES


Han, K., Hong, J. & Lim, H.C. (1993). Relieving effects of glycine and methionine from acetic acid inhibition in *Escherichia coli* fermentation. *Biotechnology and Bioengineering* **41** 316-324.


APPENDIX 1

Statistical Analysis of Dry Cell Weight Measurement

A culture of *Streptomyces lividans* TK24 was grown in a shake flask in SMM containing 30mM glucose and 15mM aspartate for 72h. Fifteen samples were processed (filtered, washed, dried and weighed; see Section 2.1.4). The results were as follows (values in g·L⁻¹):

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>3.098</td>
<td>3.473</td>
<td>2.715</td>
</tr>
<tr>
<td>3.735</td>
<td>3.180</td>
<td>2.870</td>
</tr>
<tr>
<td>2.908</td>
<td>3.023</td>
<td>2.690</td>
</tr>
<tr>
<td>3.368</td>
<td>3.353</td>
<td>2.823</td>
</tr>
<tr>
<td>3.310</td>
<td>3.143</td>
<td>2.943</td>
</tr>
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</table>

The mean value was 3.1g·L⁻¹. Statistical analysis, calculated according to Parker (1979), showed the 95% confidence limits to be 3.1±0.17g·L⁻¹. This method was therefore found to be accurate to within less than 5.3% (95% confidence limits expressed as a percentage of the mean).

The 15 filtrates obtained from this experiment were analysed by HPLC. Statistical analysis of several data points from each chromatogram showed this step to be accurate to within less than 3% (95% confidence limits expressed as a percentage of the mean).
APPENDIX 2
Statistical Analysis of Deoxyribose Determination Method

Fifteen 1mL samples were taken from the culture described in Appendix 1 and processed (Section 2.1.5). The results were as follows (values in pg·mL⁻¹):

<p>| | | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>10.128</td>
<td>8.080</td>
<td>8.416</td>
</tr>
<tr>
<td>9.102</td>
<td>9.856</td>
<td>8.821</td>
</tr>
<tr>
<td>8.608</td>
<td>9.102</td>
<td>8.354</td>
</tr>
<tr>
<td>8.334</td>
<td>9.102</td>
<td>9.379</td>
</tr>
<tr>
<td>8.576</td>
<td>9.218</td>
<td>8.205</td>
</tr>
</tbody>
</table>

The mean value was 8.9µg·mL⁻¹. Statistical analysis showed the 95% confidence limits to be 8.9±0.33µg·mL⁻¹. This method was therefore accurate to within less then 3.8% (95% confidence limits expressed as a percentage of the mean).
APPENDIX 3

Statistical Analysis of High Performance Liquid Chromatography

Repeat injections of all standards and medium components used were analysed using the relevant columns (Section 2.1.6). Raw data is not shown.

Using the Aminex HPX-87H column the retention times of organic acids and carbohydrates were found to be accurate to within less than 0.03% (95% confidence limits expressed as a percentage of the mean); and the peak areas were accurate to within less than 1.19% (95% confidence limits expressed as a percentage of the mean).

Using the Ultrasphere ODS C-18 column the retention times of amino acids were found to be accurate to within less than 0.04% (95% confidence limits expressed as a percentage of the mean); and the peak areas were accurate to within less than 0.50% (95% confidence limits expressed as a percentage of the mean).

It can be said than for the purpose of identifying and measuring the concentrations of medium components and organic acid products, HPLC analysis was accurate to within less than 0.04% and 1.19% respectively.
APPENDIX 4

Statistical Analysis of Total Carbon and Nitrogen Measurement

Fifteen 1mL samples were taken from the culture described in Appendix 1, diluted by one fifth with distilled water and analysed for total carbon (Section 2.1.10). The results were as follows (values in g·L\(^{-1}\)):

\[
\begin{align*}
1.563 & \quad 1.480 & \quad 1.389 \\
1.583 & \quad 1.512 & \quad 1.384 \\
1.515 & \quad 1.430 & \quad 1.441 \\
1.647 & \quad 1.569 & \quad 1.446 \\
1.529 & \quad 1.620 & \quad 1.504 \\
\end{align*}
\]

The mean value was 1.5 g·L\(^{-1}\). Statistical analysis showed the 95% confidence limits to be 1.5±0.04 g·L\(^{-1}\). The method was therefore accurate to within less than 3.0% (95% confidence limits expressed as a percentage of the mean).

Fifteen 2mL samples were also taken, dried in foil cups and analysed for total nitrogen (Section 2.1.10). The results were as follows (values in g·L\(^{-1}\)):

\[
\begin{align*}
0.296 & \quad 0.257 & \quad 0.212 \\
0.303 & \quad 0.249 & \quad 0.219 \\
0.276 & \quad 0.262 & \quad 0.211 \\
0.276 & \quad 0.244 & \quad 0.226 \\
0.275 & \quad 0.227 & \quad 0.230 \\
\end{align*}
\]

The mean value was 0.25 g·L\(^{-1}\). Statistical analysis showed the 95% confidence limits to be 0.25±0.02 g·L\(^{-1}\). The method was therefore accurate to within less than 6.7% (95% confidence limits expressed as a percentage of the mean). Inaccuracy in this method was probably due to loss of dried material when the foil cups were rolled into balls for loading into the analyser.