PROTEIN SYNTHESIS DURING DIFFERENTIATION OF *RHODOMICROBIUM VANNIELII* SWARMER CELLS

by

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Many thanks are due to my supervisor, Dr. C. S. Dow, for his guidance and patience during the production of this thesis. I acknowledge the financial support of the Science and Engineering Research Council.

I wish to acknowledge the help of Mr. M. Whiteside with computer programming and that of Mrs. V. J. Waddell with the raising of antisera.

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DECLARATION

I declare that this thesis has been composed by myself from the results of research conducted under the supervision of Dr. C. S. Dow and has not been used in any previous application for a degree. Furthermore, all published work has been acknowledged by reference.

ABBREVIATIONS

ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAP(CRP)	catabolite gene activator protein
cGMP	cyclic guanosine monophosphate
Ci	curie
CPM	counts per minute
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethylenediamine tetra-acetic acid
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
IF	initiation factor
M	molar
mM	millimolar
mol	mole
mmol	millimole
µCi	microcurie
µM	micromolar
µmole	micromole
mRNA	messenger RNA
NEPHGE	non-equilibrium pH gradient electrophoresis
PAGE	polyacrylamide gel electrophoresis
POPOP	1,4-bis-(5-phenyloxazole-2-yl) benzene
ppApp	adenosine-5'-diphosphate-3'-diphosphate
ppGpp	guanosine-5'-diphosphate-3'-diphosphate

PPO	2,5 diphenyloxazole
pmole	picomole
RNA	ribonucleic acid
RNAase	ribonuclease
RNP	ribonucleoprotein
r-protein	ribosomal protein
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TRIS	tris(hydroxylmethyl)aminomethane
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume

SUMMARY

The role of sequential protein synthesis in the differentiation of Rhodomicrobium vannielii swarmer cells is investigated. The cell division cycle of this organism is discussed and compared with those of other organisms both prokaryotic and eukaryotic. The advantages of using budding bacteria in general and R. vannielii in particular in the study of the control of the cell division cycle is discussed.

Evidence is presented that the obligatory maturation period of the swarmer cell before DNA synthesis begins may be correlated with the prokaryotic I period and the eukaryotic G1 phase. The length of the swarmer cell maturation period is controlled by the availability of light probably acting at the level of energy availability. A computer model based on the hypothesis that swarmer cell maturation is inhibited by low light intensity due to rising optical density as cell numbers increase accurately predicts the increase in the proportion of swarmer cells in late exponential phase.

During the differentiation of synchronised swarmer cells the pattern of protein synthesis shows many qualitative and quantitative changes indicating sequential and periodic synthesis. Some proteins are limited in their distribution to a particular cell type such as the stalked cell or swarmer cell. Protein synthesis continues in the inhibited swarmer cell and a few proteins are specific to this stage of the cell division cycle while many more are specifically synthesised during differentiation. The possible means by which sequential protein synthesis could be controlled are discussed.

An 11.5 K protein, the synthesis of which is specific to the inhibited swarmer cell, is degraded as the cell differentiates when light limitation is removed. This degradation correlates with an increase in ATP levels in the cell. The possible role of the 11.5 K protein is discussed although this is at present speculative. Antibody to this protein has been prepared making purification by affinity chromatography and gene cloning possible lines of future research.

1 INTRODUCTION

1.1 General Introduction

The regulation of cell differentiation is an important area of research in both prokaryotic and eukaryotic systems and it is possible that studies of the regulation of differentiation in prokaryotes may be extrapolated to provide clues to the nature of eukaryotic differentiation. It is probable that there are basic principles of differentiation common to all systems, both eukaryotic and prokaryotic, since at the molecular level, in the genetic code, transcription and translation, there is considerable similarity. The elucidiation of the genetic code using Escherichia coli, and its extrapolation to all organisms as it became to be regarded as near universal in its application, illustrates the usefulness of less complex prokaryotic systems. There are, however, differences between prokaryotic and eukaryotic genetic structure such as the presence of introns in eukaryotic DNA which necessitates the processing of primary transcripts to produce mature messenger RNA. This has not been demonstrated in the eubacteria but does occur in the archaebacteria (Kaine et al., 1983; Rogers, 1983) and it may be that introns have been lost during the evolution of the eubacteria (Doolittle, 1978). The control of bacterial genes by repressor proteins as in the operator-promoter model of Jacob and Monod (1961) does not occur in eukaryotes and this may be due to the presence of introns.

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It has been suggested that the perfect system to use in the study of differentiation should exhibit five major characteristics (Clarke, 1971).

1) The system should show simple and well defined morphological changes which can be caused to occur with a high degree of synchronisation.

2) The cells should be able to grow in a chemically defined medium in order to permit a better study of biochemical events associated with morphogenesis.

3) The organism should be easy to work with metabolically without major problems in making cell free extracts or in isolating enzymes.

4) The morphogenetic cycle should be subject to nutritional control and it should be possible to grow the organism through a cycle with no morphogenetic changes occurring so that changes associated with morphology can be separated from those associated with cell aging.

5) The cells should have a genetic system that can be studied by the conventional methods of bacterial genetics with stable mutants and a system for the exchange of genetic information.

Following a discussion of some of the systems of cell differentiation, the subject of this thesis, Rhodomicrobium vannielii swarmer differentiation will be considered with respect to these five criteria and compared with other systems.

1.2 Developmental Systems

A number of prokaryotic and simple eukaryotic systems have been

investigated for changes during development and differentiation. A few of the lower eukaryotes that have been studied will be discussed in order to bridge the gap between the prokaryotic systems and development in the higher eukaryotes so far studied in systems such as vertebrate cell culture, amphibian oocytes and echinoderm eggs, which will only be briefly discussed.

1.2(a) Prokaryotes

Table 1.1 contains a summary of the prokaryotic organisms to be discussed with an indication of the complexity of their life cycles, the presence or absence of specialised cells, resting cells or the existence of cell to cell interactions.

1.2(a)(i) Escherichia coli. Escherichia coli has one of the most simple cell cycles with the only morphogenesis during the normal cycle being cell division by binary fission (Gilleland and Murray, 1975). Periodic events occur during the cell cycle and include the initiation of chromosomal DNA replication, nucleoid segregation and septation. A specific event in the cell cycle may require the synthesis of a specific protein just prior to the event. If this is so, the pattern of protein synthesis through the cell cycle should change in a characteristic fashion. Using 2-dimensional polyacrylamide gels (O'Farrell, 1975) the pattern of synthesis of approximately 750 individual proteins through the cell cycle of E. coli B/r showed none that were synthesized at different rates during different stages of the cell cycle (Lutkenhaus et al., 1979). This suggests that there is no developmental programme of sequential gene expression in E. coli. Other controls suggested include changes in enzyme activity mediated by inhibitor or activator concentrations, or by enzyme location if membrane bound, and physical

Table 1.1

Life Cycle Complexity of Prokaryotes

Organism	Motile Swarmers	Resting Cells	Biochemically Specialised Cells*	Multi-cellularity	Cell-Cell Inter-Actions
Escherichia coli	-	-	-	-	-
Bacillus	-	+	-	-	-
Mycococcus	+	-	-	+	-
Nocardia	-	-	-	+	-
Geodermatophilus	+	-	-	+	-
Streptomyces	-	+	-	+	-
Arthrobacter	-	-	-	-	-
Chlorogloea	-	-	+	+	+
Anabaena	-	+	+	+	+
Myxobacteria	+	+	-	+	+
Caulobacter crescentus	+	-	-	-	-
Hyphomicrobium	+	-	-	+	-
Rhodopseudomonas spp	+-	-	-	-	-
Rhodomicrobium vannielii	+	+	-?	+	-?

+ Cell type present
- Celltype absent
+- Present in some species

* Biochemically specialised cells are for this purpose regarded as cells performing a special function during the vegetative growth of a multicellular organism.

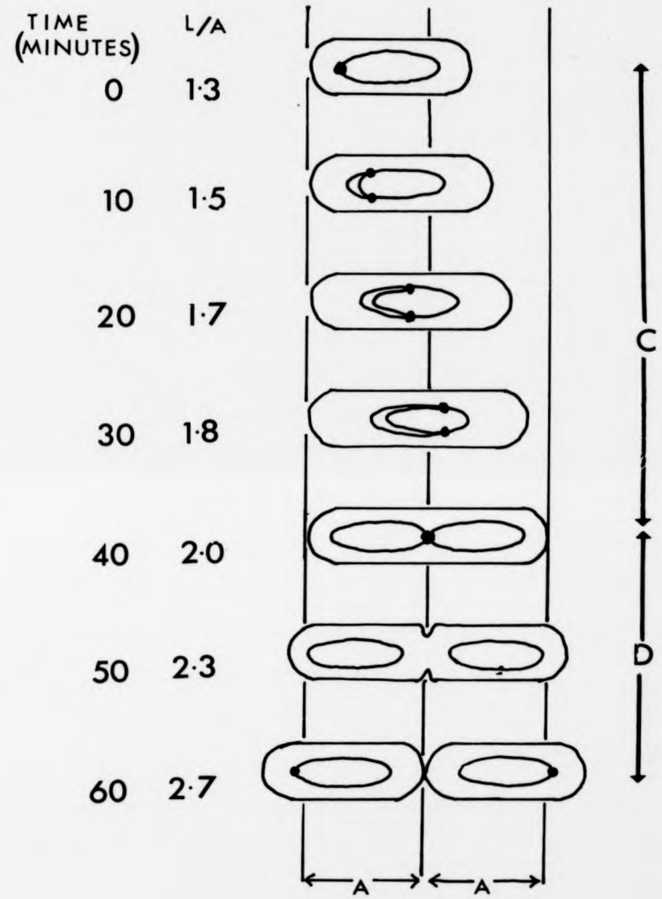
parameters such as reaching a critical cell volume or a critical concentration of a particular set of cell proteins (Lutkenhaus *et al.*, 1979).

A unit cell model was proposed in which a minimum cell length was postulated having a fixed number of elongation sites (Donachie *et al.*, 1976). These authors calculated mean cell lengths from cell length distributions of *E. coli* B/r growing exponentially in different media. It was found that $\bar{L} = (2 + 2R/3)$ μm where L is the average cell length and R is the number of generations per hour. The length of a cell at birth $L_B = \bar{L} \ln 2$ and the length at division $L_D = 2L_B$. The minimum cell length is the length at birth in a culture growing infinitely slowly so that R = 0. This "unit cell length" A = 2 ln 2 μm = 1.39 μm. At all growth rates when cells reach a length of 2A, or approximately 2.8 μm, septation is initiated between the two unit cells and the growth rate doubles as each cell begins to grow independently. This unit cell model assumes that growth rate is linear and doubles when DNA replication is terminated and septation initiated (Donachie *et al.*, 1976; Cullum and Vicente, 1978; Ward and Glaser, 1971). At doubling times of 170 mins Koppes *et al.*, (1978) found that length increased exponentially but this may have been a result o the slow growth rate. Ward and Glaser (1971) had reported linear growth with doubling at a specific stage but experimentally the curve was difficult to distinguish from exponential growth.

Chromosome replication must be co-ordinated with cell division and the C period during which DNA is synthesised varies little from 40 minutes for generation times up to 60 minutes (Helmstetter and Cooper, 1968). DNA synthesis terminates when the cell reaches a length of 2A or two unit cells and this is followed by the initiation of septation and after a 20 minute D period by cell division (Donachie *et al.*, 1976) (see Figure 1.1). If the cells are dividing more frequently than 60 minutes

Figure 1.1 (Modified from Donachie 1979)

The "Unit Cell" model of cell growth in E. coli growing with a generation time of 60 minutes. "L/A" is the cell length expressed in terms of the minimum unit cell length "A". "C" represents the period of DNA synthesis and "D" the period needed for completion of septation.



the next round of replication must start before the present division cycle is completed and with doubling times of less than 40 minutes chromosomes bear multiple replication forks because new initiations occur before the previous round of DNA replication is completed (Cooper, 1979) (see Figure 1.2). For generation times of greater than 60 minutes the C period is approximately 2/3 of the generation time while the D period remains roughly constant at 20 minutes (Davern, 1979). The division cycle of E. coli at long generation times can be described in terms of three time periods: C and D as described previously and a variable I period when initiation components are synthesized and complexes assembled to allow initiation of DNA synthesis at all chromosome origins in the cell (Helmstetter et al., 1979). The initiation of DNA synthesis has been correlated with the attainment of a particular cell mass such that 2^n rounds of chromosome replication are initiated each time the cell mass reaches $2^n.M_i$, where M_i equals twice the unit cell mass M_o (Donachie, 1968) (see Figure 1.3). At the time of initiation the cell mass (M) divided by the number of copies of the chromosome origin (O) equals M_i (Donachie, 1979).

$$M/O = M_i = 2M_o$$

For doubling times of greater than 60 minutes $M = M_i$ and $(O) = 1$. For doubling times between 30 and 60 minutes $M = 2M_i$ and $(O) = 2$. For doubling times between 20 and 30 minutes $M = 4M_i$ and $(O) = 4$. Although the initiation mass, M_i, is constant at high growth rates it is not at slower rates. The initiation mass increases about twofold when the growth rate increases from 0,6 to 1.6 doublings per hour (Churchward et al., 1981).

The average cell mass of E. coli increases as a function of the growth rate such that $\bar{M} = 2^R M_o/\ln 2$ where $\bar{M}$ is the average cell mass, R the number of generations per hour and M_o the unit cell mass. The mass

Figure 1.2 (Modified from Cooper and Helmstetter 1968)

State of the chromosomes during the division cycle of E. coli at various doubling times "T". Replication points are represented by black circles and in fact the chromosomes are circular and have a matching set moving in the opposite direction.

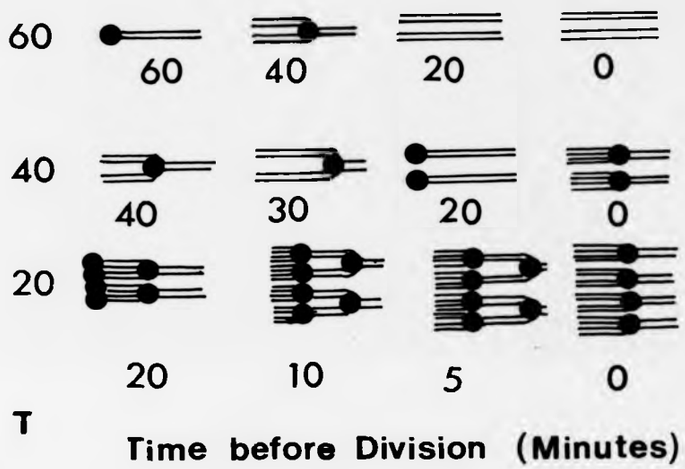
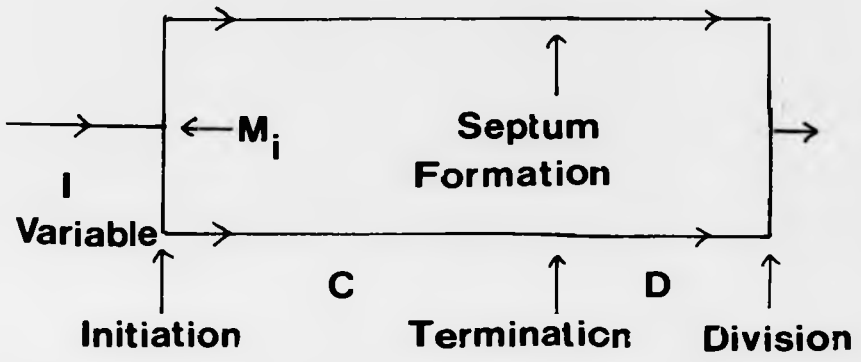


Figure 1.3 (Modified from Jones and Donachie 1973)

A cell reaching the initiation mass "Mi" initiates DNA replication and the processes leading to cell division. At the end of the "C" period, DNA synthesis is terminated and septum formation is initiated, division taking place at the end of the subsequent "D" period. During the "I" period initiation components are synthesised and complexes assembled to allow initiation of DNA synthesis at all chromosome origins in the cell.



DNA Synthesis

at birth of a cell $M_B = \bar{M} \ln 2 = 2^R M_o$ (Helmstetter et al., 1968). Combining this with the expression for the average cell length at different growth rates $\bar{L} = (2 + 2R/3)\mu M$ and assuming that cell mass is proportional to cell volume the variation of values with growth rate may be calculated if the cells are taken to be perfect cylinders (see Table 1.2).

The increase in cell radius indicates that the ratio of surface area to volume decreases with faster growth rate in richer media. In the cell cycle of E. coli the mode of growth is assumed to be elongation without change in cell diameter and to a first approximation this keeps the surface to volume ratio constant (Donachie, 1979). However, this approximation ignores the area of the two hemispherical ends. Trueba and Woldringh (1980) found that cell diameter decreased with increasing cell length and increased again during constriction and cell separation and they postulated that this was necessary to keep the surface to volume ratio constant. Considering the cell to be a cylinder with hemispherical ends of length L and radius r then:

$$\text{Area} = A = 2\Pi rL$$

$$\text{Volume} = V = \Pi r^2(L - 2r/3)$$

$$\text{Then } A/V = 2L/r\ (L - 2r/3)$$

The shortest possible cell is a sphere such that $L = 2r'$ then:

$$A'/V' = 3/r'$$

If the surface to volume ratio is to remain constant at this value then $A/V = 2L/r\ (L-2r/3) = 3/r'$

This implies that as $L \longrightarrow \infty$ in the case of filamentous growth $r \longrightarrow 2r'/3$. During constriction and cell separation the cell diameter increases to maintain the surface to volume ratio constant with extra surface area coming from the two newly formed hemispherical ends. It may be that the initiation of DNA synthesis is controlled by the

Table 1.2

Variation of Cell Radius with Growth Rate

R	M_B	L_B (μm)	r (radius)
0	M_o	2 ln 2	r_o (radius of unit cell)
1	$2M_o$	2.67 ln 2	1.22 r_o
2	$4M_o$	3.33 ln 2	1.55 r_o
3	$8M_o$	4 ln 2	2 r_o

attainment of a critical cell mass or the synthesis of initiation components sufficient for all chromosome origins present (Davern, 1979).

The replication of the DNA may be conceived as being effected by a membrane bound replication complex and the segregation of daughter chromosomes being assured by the physical attachment of one strand to a cell membrane site. The termination of chromosome replication is necessary for subsequent cell division but does not control the increase in the rate of elongation (Donachie *et al.*, 1976). Cell division and chromosome replication may be co-ordinated because of the restraints that the physical presence of a chromosome or chromosome-membrane attachment sites place on the development of a septum. Tang and Helmstetter (1980) found that the replication of a specific chromosomal region, such as the terminus, is not a mandatory requirement for cell division so it may be concluded that cell division and DNA replication follow separate pathways in *E. coli* and are co-ordinated by physical parameters such as cell volume or mass and chromosomal membrane attachment.

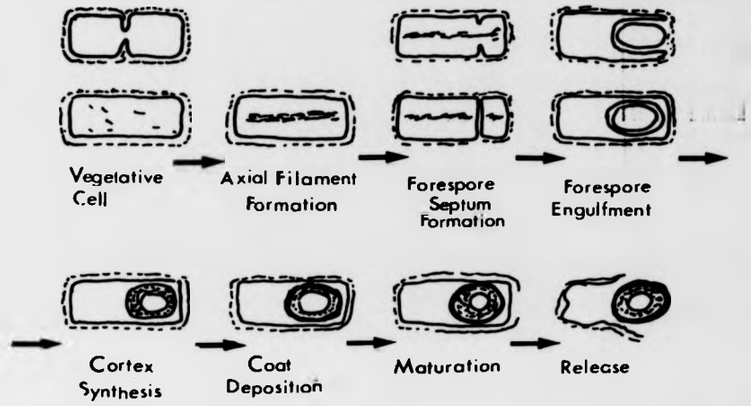
1.2(a)(ii) *Bacillus*. The differentiation of vegetative cells of *Bacillus subtilis* or *B. licheniformis* to heat resistant, refractile endospores is perhaps the most intensively studied system of bacterial differentiation. The consensus is that sporulation is triggered by starvation and this leads to the hypothesis that the presence of a carbon and nitrogen source inhibits sporulation by catabolite repression (Szulmajster, 1979). It has also been suggested that highly phosphorylated nucleotides such as Adenosine 5'-diphosphate-3' diphosphate (ppApp) and Adenosine 5'-triphosphate-3' diphosphate (pppApp) relieve repression of sporulation when their synthesis is induced by the exhaustion of nutrients (Rhaese *et al.*, 1977). The starvation stimulus must be

applied when the DNA is at a particular stage of replication about 15 minutes after initiation, i.e. when the replication fork reaches a particular site. The existence of spore-specific mRNAs suggests that sporulation is controlled at the level of transcription but 70-80% of vegetative mRNAs continue to be synthesised. Sporulating cells pass through a sequence of morphological stages from the vegetative cell, stage 0, to the free spore, stage VII (see Figure 1.4). There are mutants with blocks at various stages in this sequence which greatly facilitates the study of the genetics of sporulation (Szulmajster, 1979). The germination of spores is activated by heat treatment at 65°C for 2 hours and initiated by germinating stimulants which include amino acids, nucleosides, and glucose. Following germination the spore swells, breaks out of its integument and elongates. The outgrowth is a developmental process which is characterised by specific patterns of protein synthesis which is dependent on prior RNA synthesis and probably regulated at the level of transcription (Hansen et al., 1970).

1.2(a)(iii) Actinomycetes. The morphological manifestations of development differ greatly between surface and liquid cultures in the Actinomycetes presenting difficulties for research. The majority of the differentiated members of the Actinomycetales form aerial mycelia and spores in surface cultures, and in shaken liquid cultures a mycelial mass of marked heterogeneity is produced. There is a great variation in the complexity of development amongst members of the Actinomycetales. A number of species do not produce specialised reproductive cells and vegetative reproduction is by cell septation and budding. The simplest development is illustrated by Mycococcus which forms neither mycelial or rodlike forms and undergoes irregular division resulting in cells of uneven shape and size. In Nocardia and many other actinomycetes

Figure 1.4 (Modified from Hanson et al. 1970)

Stages during sporogenesis in Bacillus spp.



vegetative reproduction is by mycelial fragmentation with multiple septa dividing the growing hyphae more or less regularly (see Figure 1.5). Those actinomycetes that retain unfragmented mycelia during large proportions of their life cycle such as Streptomyces usually reproduce by asexual sporulation. The spores may be formed on substrate and/or aerial mycelia and either as single cells or in chains. Little progress appears to have been made with the actinomycete development systems due to the heterogeneous nature of most cultures. However the group does represent a higher level of prokaryotic complexity and could repay further study.

Geodermatophilus is a genus of the Actinomycetales which exists in two major forms (Ishiguro and Wolfe, 1970). The R form is a motile rod which reproduces by budding and the C form is a non-motile irregular aggregate of cocci. Morphogenesis can be controlled by an unidentified factor M in tryptone which is needed to maintain the culture in the C form and convert the R form to the C form (see Figure 1.6). The C form reproduces by binary fission and aggregates divide on reaching a maximum size. If C form cells are transferred to a medium lacking the factor M small buds appear on the coccoid cells in a few hours and the R form is released by fission. The C form cells are incapable of reproducing in the absence of the factor M and ultimately lyse. The motile R form cells have from one to four flagella and the buds develop on slender stalks which are retained after fission. If the R form is transferred to a medium containing the factor M the cells enlarge and produce storage granules. Transverse and longitudinal septa are produced giving elongated cell aggregates initially. A variety of inorganic cations such as Na^+, K^+, Rb^+, Ca^{++}, Mg^{++}, NH_4^+ induce and maintain the C form, in concentrations above 100 mM (Ishiguro and Wolfe, 1974). The uptake of cations is accompanied by the extrusion of intracellular H^+ producing

Figure 1.5 (Modified from Kalaboutski and Agre 1976)

a) Transverse septation in Mycococcus vegetative cells.

b) Division in hyphae of Nocardia.

c) Septation in sporulating hyphae of Streptomyces.

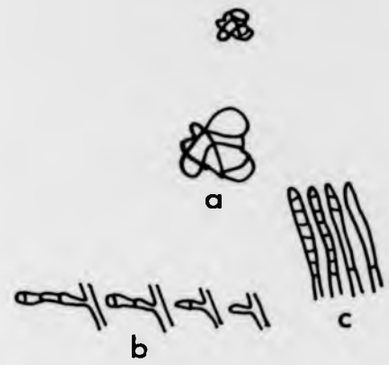
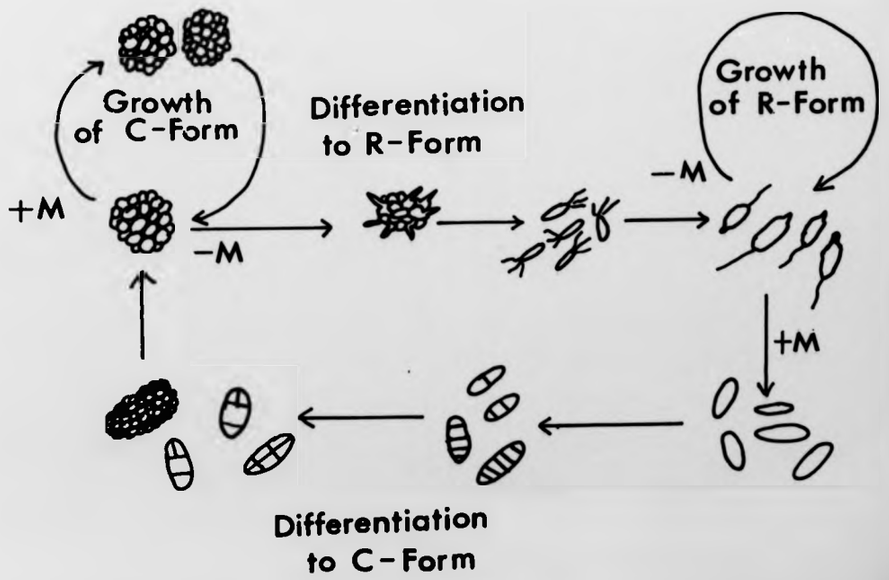


Figure 1.6 (Modified from Ishiguro and Wolfe 1970)

Morphogenesis in the growth cycle of Geodermatophilus.



a rise in intracellular pH. Organic amines such as methylamine and dimethylamine are also taken up and induce a rise in intracellular pH and also morphogenesis. It seems therefore that it is the rise in intracellular pH that is the primary cause of the morphogenetic switch from the R form to the C form. Such a rise in pH can have a profound effect on metabolism as for instance in Streptococcus faecalis where glycolysis is inhibited by low intracellular pH and restored by uptake of NH_4^+, K^+, Na^+ or organic amines (Tyler and Marshall, 1967).

1.2(a)(iv) Arthrobacter. Arthrobacter crystallopoietes is another organism with morphologically distinct growth forms in different media (Ensign and Wolfe, 1964). Cells in the stationary phase of growth are spherical but on inoculation into fresh medium gradually elongate to produce pleiomorphic rods. Cell division occurs in the rod stage and continues until the rods break into shorter cells and eventually return to the coccoid form in the stationary phase (see Figure 1.7). Reversion to the coccoid form may be connected with survival in dry soil since the coccus has the smallest surface to volume ratio. On a synthetic glucose-salts medium there is a longer lag period and slower growth but a similar cell density to that found in complex media is reached. However on synthetic media the rod stage is not developed and cells grow and divide in the coccoid stage. Various substances added to the glucose-salts medium induce the appearance of the rod stage (Ensign and Wolfe, 1964). These substances include amino acids such as L. phenylalanine, L. asparagine, L. lysine and L. arginine and other organic compounds such as butyrate, succinate, malate, fumarate and lactate. All TCA cycle intermediates with the exception of citrate result in the induction of the rod stage. Succinate and several other compounds that induce the sphere to rod morphogenesis suppress the

Figure 1.7 (Modified from Clark 1979)

Morphogenetic changes in Arthrobacter.

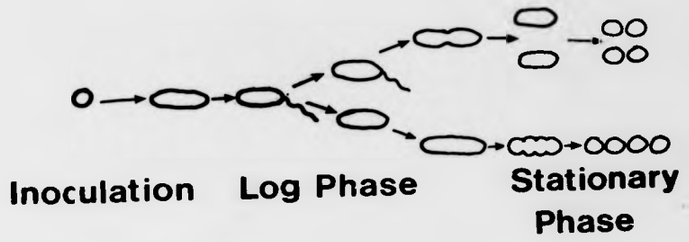
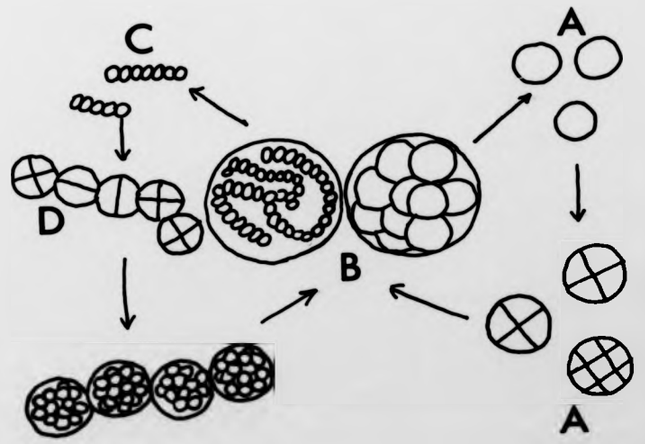


Figure 1.8 (Modified from Evans et al. 1976)

Cell types found during photoautotrophic growth of Chlorogloea fritschii showing their interrelationships.



catabolism and assimilation of glucose and diauxic growth patterns result from growth on glucose plus succinate with a lag after the exhaustion of succinate while a repressed enzyme is synthesised to catabolise glucose. The control of morphogenesis may lie in metabolic control but it is difficult to see a common mechanism for the compounds found to be active and close analogues of the inducers fail to affect the growth cycle. The morphogenetic cycle involves changes in cell shape suggesting parallel changes in cell wall composition. The polysaccharide backbones of the peptidoglycan in spherical cells is heterogeneous in length averaging less than 40 hexosamines per chain while those in the walls of rod cells are more homogeneous and about 3 times as long (Krulwich et al., 1967a). The sphere to rod transition may involve alteration of enzymes that produce the peptidoglycan or of an autolysis that hydrolyses glycan (Krulwich et al., 1967b). Changes in activity of an autolytic N-acetyl-muramidase during sphere-rod morphogenesis correlates with the changes in polysaccharide backbone the activity being low during the rod stage when the polysaccharide backbone is longer than in the sphere stage (Krulwich and Ensign, 1968). Compounds which produce the sphere to rod morphogenesis suppress glucose metabolism which is a poor substrate for A. crystallopoietes (Krulwich and Ensign, 1969). Glucose permease is inhibited by succinate and its synthesis suppressed in succinate grown cells.

A. crystallopoietes is unique in the genus in that most compounds that support growth do not induce the rod stage (Lucas and Clarke, 1975). In general when a compound supports growth it also induces transition to the rod stage as seen with 38 different compounds tested amongst 17 species of Arthrobacter.

A. globiformis undergoes the sphere to rod transition in cation complete medium reverting by reductive fragmentation at the onset of the

stationary phase (Germida and Casida, 1980). The addition of metal complexing agents causes growth in a myceloid form consisting of long filaments with rudimentary branching. Manganese appears to be the critical ion removed. The occurrence of transitory myceloid growth in some complex media may be due to the presence of chelators in the medium such as proteins or amino acids (Germida and Clark, 1980). In the later stages of cultures with a high carbon to nitrogen ratio cells may be transformed into enlarged oval or lemon shaped cells called cystites (Duxbury et al., 1977). Cystites may form from rods as well as cocci and have been described as senescent forms, cells with a reproductive function, or cells with abnormal accumulations of storage compounds (Duxbury et al., 1977). Cystites are produced in unbalanced conditions and are capable of reverting to normal cells on restoration to complete media (Duxbury and Gray, 1977). Thus the genus Arthrobacter offers, from the viewpoint of morphogenesis, marked changes during the cell cycle in response to changes in media constituents.

1.2(a)(v) Chlorogloea fritschii. The cyanobacterium Chlorogloea fritschii exists in more than one morphologically recognisable form depending on the environmental conditions (Evans et al., 1976). If C. fritschii is grown photoautotrophically, with nitrate present as the nitrogen source, four cell types appear in the exponential phase (see Figure 1.8). Type A cells are large granulated cells in small groups or singly. Type B cells are found in large clumps surrounded by a mucilaginous sheath. Type C cells are small and in short filaments while type D cells are longer and found in dividing filaments. Photoheterotrophic growth in the presence of sucrose leads to the predominance of cell type B. Deprivation of sucrose or elevated temperature leads to the typical mixed appearance of photoautotrophic

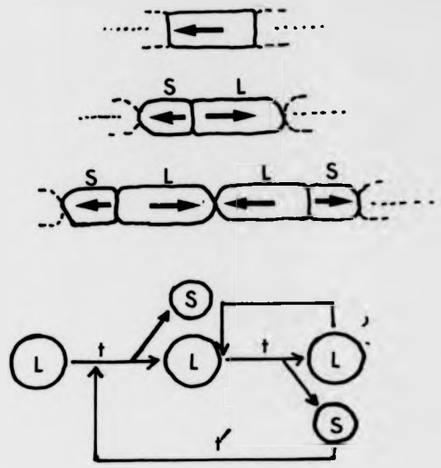
growth with the filamentous forms finally becoming dominant. In addition to these changes the deprivation of nitrate leads to the development of heterocysts as in other cyanobacteria (Evans et al., 1976).

1.2(a)(vi) Anabaena. Heterocysts are specialised nitrogen fixing cells produced by cyanobacteria when exogenous free nitrogen is depleted and in Anabaena catenula they appear along the filament at intervals to form a spaced arrangement (Mitchison and Wilcox, 1972; Wolk, 1982). The filament grows by the division of all its vegetative cells but the heterocysts do not divide. The spacing between heterocysts is maintained by the differentiation of a vegetative cell to a heterocyst midway between existing heterocysts. When the vegetative cells divide one daughter cell is always smaller than the other at the time of division because septum formation always takes place more than half a cell length from the previously existing and most recently produced septum. The small daughter cell takes longer to divide than the large daugher cell resulting in an asynchronous population down a filament of vegetative cells (see Figure 1.9). Heterocysts only develop from the small daughter cells but there is an inhibitory zone of 4 to 5 cells around each heterocyst preventing the development of nearby small cells into heterocysts (Mitchison and Wilcox, 1972).

Most small daughter cells which at the time of their formation are within 5 cells of a heterocyst or a developing proheterocyst fail to differentiate. However those small daughter cells that are 5 or more cells distant from a heterocyst or proheterocyst almost invariably differentiate (Adams and Carr, 1981). New proheterocysts are placed symmetrically with respect to existing heterocysts either exactly midway or as a pair with at least 3 vegetative cells between them. At a

Figure 1.9 (Modified from Adams and Carr 1981)

The division rule in Anabaena. In each cell an arrow points away from the last newly formed septum and septum formation always takes place more than half a cell length from this producing large and small daughter cells. The small daughter requires a longer division time (T') than the larger daughter (t) resulting in an asynchronous population of cells along the filament.



filament end proheterocyts seldom develop in the last two or three cells and very rarely indeed in the terminal position. A simple model to explain the placement of proheterocysts assumes that a proheterocyst produces an inhibitor to which it remains susceptible and may regress if the inhibitor rises above a critical level. As the proheterocyst develops the critical inhibitor level rises and it also produces inhibitor at an increasing rate. In trying to express these ideas algebraically one may assume that "x" is a substance whose concentration increases with proheterocyst development and does not diffuse and that "y", the inhibitor, does diffuse and its rate of synthesis depends on "x" (Wilcox et al., 1973). Thus $d[y]/dt = A[x]$ and $d[x]/dt \gtrless 0$ dependent on whether $[x] \gtrless B[y]$. If the background level for [x] in vegetative cells equals "L" then when [y] falls below L/B, $[x] > B[y]$ so that $d[x]/dt > 0$ differentiation can begin. The model assumes that the inhibitor "y" is lost from or destroyed in some way in vegetative cells. Two nearby proheterocysts cause an increase in [y] in each other so preventing development within a critical distance. One may also assume that small daughter cells produce less "y" than large daughter cells so they are more likely to differentiate. However there is no direct evidence for the existence of "x" and "y" so the model is little more than surmise. It also fails to explain why the end cell of a filament rarely differentiates as it should have the lowest concentration of inhibitor of any cell in the vicinity.

It is possible that the pattern of heterocyst differentation is partly a consequence of the asynchronous cell cycles caused by the unequal cell division. Heterocyst formation has a light dependent stage followed by a light independent stage and if vegetative cells have to be at a certain stage of the cell cycle in order for differentiation to be triggered by light the varying ages of cells along a filament could

ensure that heterocysts are distributed symmetrically (Bradley and Carr, 1977; Carr, 1979).

Some, but not all, species of Anabaena also produce resting cells called akinetes probably induced by incipient desiccation or depletion of nitrogen or phosphorus but no defined environmental trigger has been discovered (Adams and Carr, 1981). In Anabaena cylindrica akinete formation occurs at the end of exponential growth with a gradient of maturity away from a heterocyst (Adams and Nichols, 1982). Cells that are to become akinetes enlarge and elongate and form a fibrous coat between the cell wall and the mucilaginous sheath that encloses the filament. Akinetes presumably serve as survival cells and there is evidence of a greater resistance to cold and desiccation than vegetative cells (Adams and Nichols, 1982).

1.2(a)(vii) Myxobacteria. The myxobacteria are gram-negative bacteria possessing gliding motility and are unique among bacteria in having a life-cycle with cellular and colonial morphogenesis (see Figure 1.10; Kaiser et al., 1979). Most of the species are bacteriolytic and produce extracellular proteases, lipases, and nucleases. The vegetative rods grow and divide by binary fission with a generation time of about 210-270 minutes. When the medium becomes depleted of specific amino acids on a solid medium the cells aggregate and form fruiting bodies. The cells become round, refractile, resistant myxospores. Sporulation can be induced by 0.5 M glycerol with the absence of fruiting body formation. The regular sequence of morphological changes seen in the formation of fruiting bodies implies underlying biochemical changes. About one-quarter of the proteins analysed by gel electrophoresis show significant changes during fruiting body formation (Kaiser et al., 1979). Mutants have been obtained that suggest that there are

Figure 1.10 (Modified from Kaiser et al. 1979)

The myxobacterial vegetative and developmental cycles.

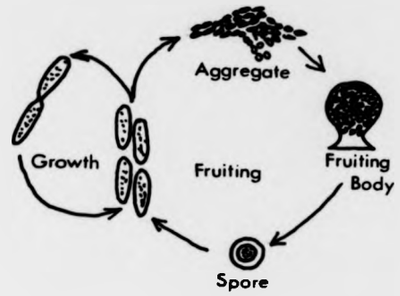
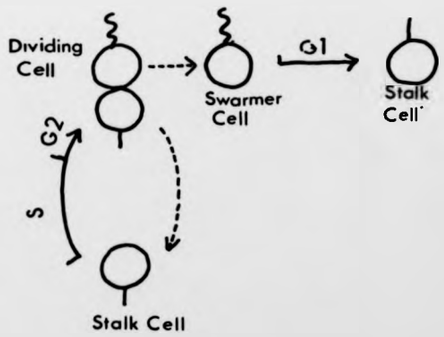


Figure 1.11 (Modified from Osley and Newton 1977)

Cell cycle of Caulobacter crescentus showing the maturation phase (G1) before a swarmer cell can initiate DNA synthesis while a stalk cell can initiate DNA synthesis immediately following division.



developmental steps upon which both aggregation and sporulation depend, some steps upon which only aggregation depends, and some on which only sporulation depends (Kaiser et al., 1979). It seems that fruiting can be divided into three parts; initiation by starvation, aggregation including fruiting body formation, and myxospore formation.

1.2(a)(viii) Caulobacter crescentus. In Caulobacter crescentus the cell cycle leads to the production of two cell types, a flagellated swarmer and a stalked mother cell. The swarmer cell must differentiate to a stalked mother cell before DNA synthesis can be initiated but the mother cell does not need such a maturation phase. This period is equivalent to the G1 phase of the eukaryotic cell cycle (see Figure 1.11; Osley and Newton, 1980). C. crescentus undergoes microscopically distinct events through the cell cycle and has been much used in studies of bacterial differentiation. The dimorphic cell cycle of C. crescentus contrasts with that of E. coli in which division produces cells that are microscopically identical. A number of proteins synthesized during the development of C. crescentus are made either periodically at defined periods in the cell cycle or continuously in only one of the two cell types, which is in contrast to the situation found in E. coli (Sheffery and Newton, 1981; Milhausen and Agabian, 1981; Ohta et al., 1982).

The polar surface structures; flagella, pili, phage receptors, and stalk are under positional and temporal control in the cell cycle (Fukuda et al., 1981). The formation of these structures can be blocked by a single mutation suggesting a common control mechanism.

Chromosome replication has been considered to act as a cell cycle clock with the initiation and completion of the division pathway leading to cell division controlled by successive stages of DNA synthesis. The temporal order of morphological changes is achieved by late steps being

dependent on the completion of earlier steps (Osley and Newton, 1980). For instance, stalk formation is dependent on prior cell division and subsequent cell division on stalk formation so that unless the swarmer cell differentiates into a stalked, cell DNA synthesis and cell division do not occur.

There is some evidence in Caulobacter that the control of the assembly of polar structures is separate from the control of subunit synthesis (Smit and Agabian, 1982). The pili in C. crescentus are assembled during the predivisional and swarmer cell stages and disappear as the swarmer cell differentiates into a stalked cell (Smit and Agabian, 1982). An immune precipitation assay for pilin, the protein subunit of the pilus, shows that pilin synthesis begins in the early stalked cell and is complete before cell division so that the entire period of pilin synthesis is over before pili are visible at the differentiated cell pole (Smit and Agabian, 1982).

1.2(a)(ix) Hyphomicrobium. The genus Hyphomicrobium contains species which are all chemo-organotrophs with a limited range of carbon and energy sources for growth (Harder and Attwood, 1978). All the species are aerobic but may use nitrate as terminal electron acceptor. The genus is widely distributed being found in soil, sea and freshwater. Some strains are able to form a permanent attachment to surfaces and have been observed in habitat attached to fungi and algae and they often form a surface pellicle in undisturbed laboratory cultures (Moore, 1981). Manganese oxidising hyphomicrobia have been reported to cause deposition of manganese in freshwater pipes, the bacteria having repeatedly branched prosthecae forming a network ramified through the deposit (Tyler and Marshall, 1967; Tyler, 1970). Bizarre cell shapes seen in these multicellular networks appear to be a consequence of the

production of several prosthecae from different parts of the cell.

The growth of hyphomicrobia is always polar and a bud is formed at the end of a prostheca which develops 1 to 3 polar or subpolar flagellae. This daughter swarmer cell loses motility and produces a prostheca from the pole opposite to where it was originally attached to the mother cell (see Figure 1.12). The mother cell retains the prostheca and can initiate DNA synthesis immediately after the detachment of the daughter swarmer cell while the swarmer cell requires a period of maturation corresponding to the G1 period in the eukaryotic cell cycle before DNA synthesis is initiated. This maturation period may also be compared with the I period defined for E. coli in 1.2(a)(i) (Wali et al., 1980).

1.2(a)(x) Rhodospirillaceae. The Rhodospirillaceae, which comprises the purple non-sulphur photosynthetic bacteria, contains several examples of budding bacteria with a range of morphological and cell cycle complexity as well as forms which do not reproduce by budding. The genus Rhodopseudomonas comprises 3 morphologically different species groups (Pfennig, 1977; de Bont et al., 1981):

(i) R. capsulata, R. sulfidophila, R. sphaeroides and R. globiformis are short rods, with vesicular intracytoplasmic membranes and do not multiply by budding.

(ii) R. gelatinosa has slender rod shaped to spirilloid cells with the photosynthetic apparatus consisting of the cytoplasmic membrane with small tubular or lamellar intrusions. This species closely resembles Rhodospirillum tenue and could perhaps be included in the same genus.

(iii) R. palustris, R. viridis, R. sulfidoviridis, R. acidophila, and R. blastica have rod shaped cells that multiply by budding and an intracytoplasmic membrane system of membranes parallel to, and

Figure 1.12 (Modified from Harder and Attwood 1978)

The Hyphomicrobium cell cycle.

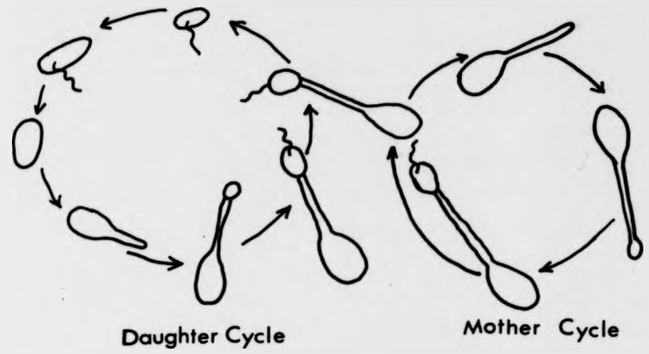
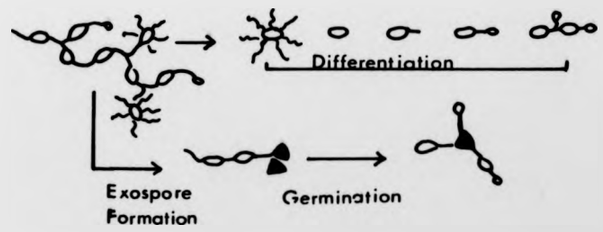


Figure 1.13 (Modified from Whittenbury and Dow 1977)

Cell types expressed by Rhodomicrobium vannielii.



underlying, the cytoplasmic membrane. This membrane system is similar to that of Rhodomicrobium vannielii which has the most complex cell cycle of the Rhodospirillaceae with ovoid cells joined by prosthecae forming multicellular arrays which also produce flagellated swarmer cells and in some strains heat- resistant exospores (see Figure 1.13; Whittenbury and Dow, 1977).

From the size and chain insertions found in their cytochromes C_2 and C_{551} Rm. vannielii appears to be closely related to Rhodopseudomonas viridis and R. acidophila but more distantly related to R. palustris, R. capsulata and R. sphaeroides (Dickerson, 1980). It is also suggested that Rm. vannielii may be related to the eukaryotic mitochondrion (Dickerson, 1980). There are however limitations in the usefulness of small macromolecules such as cytochrome c in genealogical measurement because of selected and therefore rapid changes in sequence. A large macromolecule such as 16S rRNA is less susceptible to these problems and oligonucleotide mapping shows that Rm. vannielii is closely related to R. viridis and R. palustris and more distantly to R. sphaeroides and R. capsulata (Woese et al., 1980). In either event it appears that Rm. vannielii is a close relative of the group of rhodopseudomonads that reproduce by budding and it has been suggested that these species be included in Rhodomicrobium (Pfennig, 1977). This group of budding bacteria show increasing complexity in their cell cycles culminating in Rm. vannielii (see Table 1.3 and Figure 1.14).

A relatively recently described species R. blastica (Eckersley and Dow, 1980) represents a simple stage. The cells are ovoid to rod shaped and multiplication is by budding without the production of a prostheca between the mother and daughter cells. Cell division is symmetrical and both cells are non-motile but mother and daughter are physiologically distinct with the membrane system being formed de novo in the daughter.

Table 1.3

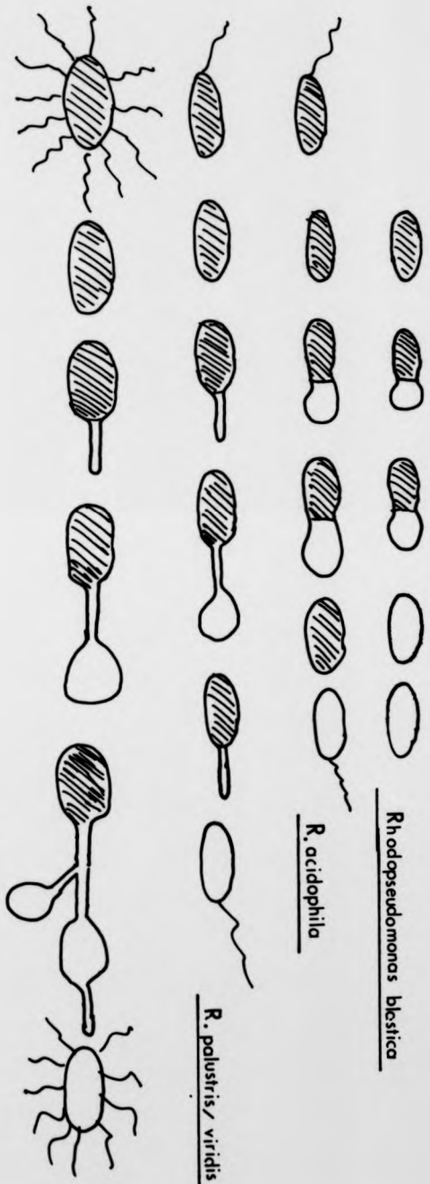
Cell Cycle Complexity of Rhodomicrobium and Related Bacteria

Organism	Motile Swarmers	Prostheca present	Multi-cellularity	Exospores
Rhodopseudomonas blastica	-	-	-	-
Rhodopseudomonas acidophila	+	-	-	-
Rhodopseudomonas palustris/viridis	+	+	-	-
Rhodomicrobium vannielii	+	+	+	+

+ = Present
- = Absent

Figure 1.14 (Modified from Whittenbury and Dow 1977)

Increasing complexity in the cell cycles of the budding bacteria related to Rhodomicrobium vannielii.



R. acidophila shows almost symmetrical division with sessile buds not formed at the end of prosthecae but these buds produce motile swarmer cells (Pfennig 1969). In the case of R. palustris and R. viridis there is production of a prostheca prior to bud formation similar to that produced by Hyphomicrobium but thicker and shorter in proportion to the cell. Reproduction in R. palustris always occurs at what may be termed the anterior pole while a holdfast and flagellum are produced at the posterior pole (Whittenbury and Dow, 1977; Whittenbury and McLee, 1967). So the pole of the daughter cell originally attached to the mother cell's prostheca eventually produces a prostheca itself.

At the extreme end of the spectrum of complexity, Rm. vannielii has ovoid cells joined by prosthecae which may be branched thus forming ramifying multicellular arrays of cells (see Figure 1.15). The cells in the arrays are separated by cross walls laid down in the prosthecae. Non stalked peritrichously flagellated swarmer cells are produced in the exponential phase and in some strains angular heat resistant exospores are produced in the stationary phase (Gorlenko et al., 1974). When the swarmer cell develops the flagellae are lost and prosthecae are produced at one or both poles but only one bud is produced at a time irrespective of the number of prosthecae. A second daughter cell may form on a prostheca branching from the first and so on to a maximum of four daughter cells even if the original swarmer cell differentiated with bipolar filaments. As these daughter cells develop the multicellular array is produced. At some stage in the development of the array swarmer cells are produced which develop to give new multicellular arrays until late in the exponential phase when the development of swarmer cells is inhibited by low availability of light as the optical density of the culture increases. This results in an increase in the proportion of swarmer cells in the culture (Dow and Whittenbury, 1979).

Figure 1.15 (Modified from Whittenbury and Dow 1977)

Differentiation and development of a Rhodomicrobium vannielii swarmer cell to produce a multicellular array.

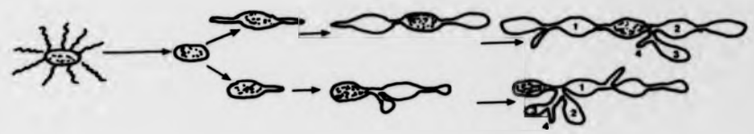
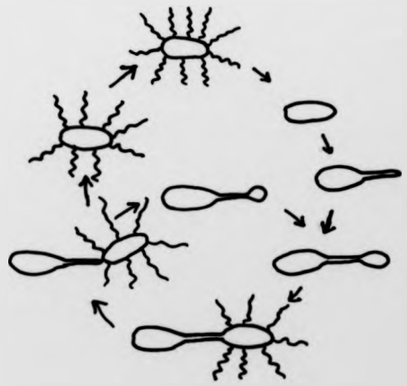


Figure 1.16 (Modified from Dow and France 1980)

Cell cycle in Rhodomicrobium vannielii expressing the "simplified" vegetative cell cycle.



The differentiation of a swarmer cell into a stalked cell and the production of a daughter cell may be divided into two periods:

(i) Maturation during which motility is lost as flagellae are shed but no other obvious morphogenesis occurs.

(ii) Reproduction during which a prostheca is synthesised. At one or both poles a bud is synthesised and the daughter is isolated from the mother cell by plug formation. Plug formation is probably under the control of the daughter cell as the distance from the daughter cell to the plug is constant irrespective of the prosthecal length (Whittenbury and Dow, 1977). If the daughter cell is released as a motile swarmer fission occurs at the junction of the prostheca and daughter cell which thus retains no part of the mother cell prostheca.

Whether a daughter cell develops into a motile swarmer or becomes part of the multicellular array may itself be under environmental control such as decreased light intensity from shading by increased cell numbers or increased carbon-dioxide tension in the multicellular arrays (Dow and Whittenbury, 1979). Both these effects are functions of cell number, consequently as with the inhibition of swarmer differentiation, the production of swarmers may be dependent on the growth phase in heterogeneous batch culture.

Some strains of Rm. vannielii exhibit a "simplified" cell cycle in which the differentiated swarmer cell immediately gives rise to a swarmer cell so that a multicellular array is not produced (Dow and France, 1980; see Figure 1.16). In this case the cell cycle is similar to that shown by the close relatives of Rm. vannielii such as R. viridis and R. palustris (Whittenbury and Dow, 1977).

1.2(a)(xi) Diversity of prokaryotic cell cycles. Prokaryotes are often regarded as inherently unicellular with cells that are totally

independent of each other. However in the actinomycetes and cyanobacteria increasingly complex multicellular prokaryotes are seen with specialised cells and interdependence between cells in a complex. The myxobacteria show cell-cell interactions and corporate behaviour in the developmental cycle and perhaps also in growth (Kaiser et al., 1979). The morphogenesis of Arthrobacter, Geodermatophilus and Chlorogloea illustrate changes in the vegetative structures that can occur in response to environmental changes and which presumably allow for maximal exploitation of resources. Also in response to environmental change resting cells may be produced such as Bacillus endospores and cyanobacterial akinetes whose function is to survive conditions that are lethal to the vegetative cell. Some prokaryotes, such as Rhodomicrobium, Hyphomicrobium and various actinomycetes are multicellular without as far as is known having biochemically specialised cells such as heterocysts or exhibiting cell-cell interactions (see Table 1.1). Multicellularity in these organisms may be a response to growth on surfaces and this could be the first step in the evolution of multicellular organisms.

The production of motile swarmers is in most cases a response to the need for a dispersal cell from the surface growing multicellular arrays or the sessile holdfast attached cells of Caulobacter. In the case of Caulobacter and some Rhodopseudomonas spp. the production and differentiation of swarmer cells is an integral part of the cell cycle while Hyphomicrobium and Rhodomicrobium may grow without swarmer production, at least in the natural environment where they are attached to surfaces. In laboratory cultures Hyphomicrobium normally exhibits a "simplified" cycle without producing multicellular arrays and Rhodomicrobium can also do so (see Figs. 1.12 & 1.16). The budding mode of growth allows the extensive membrane systems of these bacteria to be

maintained without disruption at cell division and are formed de novo in the daughter cell.

Budding is a form of polar growth and it is probable that the distinction between budding bacteria and other forms such as E. coli is not as great as appears at first sight as these bacteria may also grow polarly at least when growing slowly (Whittenbury and Dow, 1977).

1.2(b) Eukaryotes

Table 1.4 contains a summary of the eukaryotic organisms discussed in this section with an indication of the complexity of their life cycles and development.

1.2(b)(i) Mucor. The fungus Mucor shows dimorphism, growing in the presence of a hexose as a yeast with an atmosphere of 100% carbon-dioxide but as hyphae with 100% nitrogen or air (Orlowski and Sypherd, 1977). The morphological change of yeast to hyphae leads to an abrupt increase in the rate of protein and mRNA synthesis (Orlowski and Sypherd, 1977). Two dimensional polyacrylamide gel electrophoresis showed that of 400–500 proteins resolved few were specific to the yeast form or to hyphae but a large number were regulated quantitatively during the morphological conversion (Hiatt et al., 1980). Changes in the rate of protein synthesis alone can alter the ratios of gene products and the authors suggest that the small number of proteins specific for either morphological form implies that cellular morphogenesis may not necessarily be the result of dramatic differential gene expression (Hiatt et al., 1980). The increase in protein synthesis seen on transition from the yeast form to the hyphal form was found to be due to an increased velocity of ribosome movement along the mRNA during morphogenesis (Orlowski and Sypherd, 1978).

Table 1.4

Life Cycle Complexity of Eukaryotes

Organism	Resting Cells	Single Cell Stage	Separate Germ Line	Specialised Cells	Cell-Cell Interactions	Dimorphism
Mucor	+	+	-	-	-	+
Candida	-	+	-	-	-	+
Volvox	-	-	+	+	+	-
Cellular Slime Moulds	+	+	-	+	+	-
Saccharomyces	+	+	-	-	+	-
Higher Animals	-	-	+	+	+	-
Higher Plants	+-	-	+	+	+	-

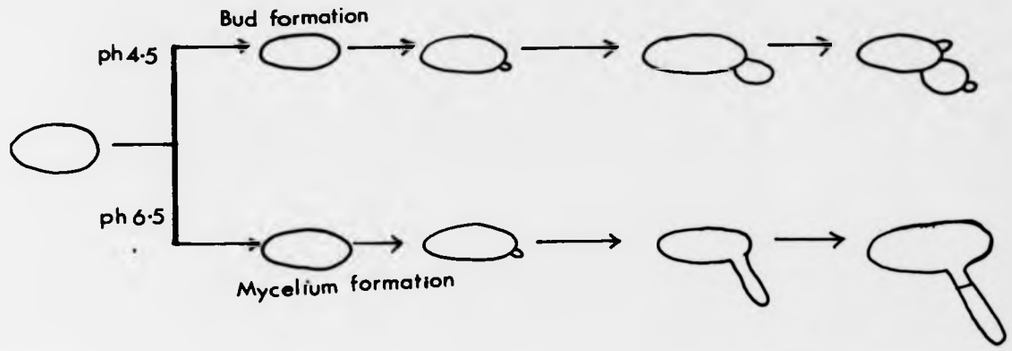
+ = Cell type present
- = Cell type absent
+- = Present in some species

The germination of spores of Mucor species has also been studied (Orlowski, 1980). The internal pools of cyclic AMP change during development of spores of Mucor genevensis and Mucor mucedo (Orlowski, 1980). Spherical growth of the spores is characterised by a large internal pool of cAMP whereas germ tube emergence and elongation of hyphae is characterized by much smaller pools of cAMP. Adenylate cyclase activity reaches a maximum during spherical growth of the spores but cAMP phosphodiesterase levels remain constant throughout germination suggesting that internal cAMP levels may be controlled by changes in adenylate cyclase activity. The addition of cAMP at the time of inoculation inhibits normal hyphal development suggesting a role for cAMP in the regulation of development in germination. cAMP also reacts specifically with a few soluble proteins whose synthesis changes in a characteristic pattern during germination and which may be of regulatory significance to development (Orlowski, 1980).

1.2(b)(ii) Candida albicans. Candida albicans can grow in either a budding yeast form or as a mycelial form in response to alterations in the medium (Brummel and Soll, 1982). When stationary phase cells are diluted into fresh medium at either pH 4.5 or pH 6.5 they form evaginations after the same lag period and in either case a high degree of synchrony. However at pH 4.5 the cells continue to grow in the budding yeast form while at pH 6.5 cells grow in an elongate mycelial form (Brummel and Soll, 1982) (see Figure 1.17). Three phases of protein synthesis were seen in both cases: Phase I with a zero rate of incorporation of labelled amino acids: Phase II with a slow rate of incorporation of radioactive amino acids: Phase III showing a dramatic increase in incorporation of radioactivity. The proteins synthesized during Phase III in both budding yeast and mycelial cultures were

Figure 1.17 (Modified from Brummel and Soll 1982)

Growth of Candida albicans in either a yeast form or a mycelial form depending on the pH of the medium.



analyzed using two dimensional polyacrylamide gel electrophoresis and it was found that the major proteins synthesized by each growth form were similar. The authors speculated that very minor form-specific proteins may be synthesized which are not distinguished by one and two-dimensional electrophoresis but raise the possibility that the regulation of dimorphism in Candida albicans may depend on mechanisms other than differential gene expression (Brummel and Soll, 1982). For example the pH of the medium may in some unknown way control the synthesis of the form specific structures.

1.2(b)(iii) Tremella. The heterobasidiomycetous yeast Tremella mesenterica secretes a mating hormone Tremerogen A-10 which induces sexual differentiation (Hayakawa and Fukui 1980). Using two-dimensional gel electrophoresis the authors found that one particular protein increases while another decreases during differentiation. This suggests that sexual differentiation results from genes controlled by the extracellular information, i.e. the mating hormone (Hayakawa and Fukui, 1980).

1.2(b)(iv) Volvox. The eukaryotic algae belonging to the genus Volvox are the most primitive organisms in which there is a morphological and functional differentiation between germ-line and somatic cells (Jaenicke, 1982). Volvox spp are composed of Chlamydomonas-like biflagellate cells which in great numbers make up a globular outer hull or syncytium. When Volvox reproduces vegetatively the primordial stem cell or gonidium divides mitotically several times resulting in a bowl like structure with gonidial cells outside and a syncytial cluster of somatic cells inside. The somatic cells flow through the "phialopore" in the centre of the gonidial assembly and form a globe of cells around

it in a process called "inversion". The gonidia develop into daughter spheroids which are released when the parent globe of cells around them is dissolved enzymatically. The vegetative cycle may be repeated indefinitely or until sexual reproduction is induced.

Volvox forms male and female gametes either in one parent spheroid or in separate spheroids. In females the morphology is similar to vegetative reproduction and the inverted gonidia form eggs, but in male gonidia the differentiation into sperm mother cells and somatic cells is delayed until the final division resulting in an equal number of each of either 128 or 256 according to the species. The sperm mother cells divide rapidly to form discoid sperm packets of 32 or 64 sperm cells. The zygote resulting from fertilization of the eggs develop a hard brown envelope and is the resting or overwintering stage. When the resting zygote germinates meiosis takes place followed by mitosis to yield new vegetative spheroids. The differentiation by unequal division into somatic and gonidial cells occurs at different stages in vegetative and sexual reproduction. With Volvox carteri determination occurs at the 32 cell stage in vegetative reproduction, at the 64 cell stage in female cells, and not until the final division in males. Thus sexual reproduction involves a delay in the determination of whether cells will be somatic or germ plasm. Sexual reproduction is under the control of an inducer glycoprotein excreted into the medium which slows down gonidial development and delays differentiation. What controls the release of the inducer is not known and it is speculated that it may be a frequent mutation in males with a frequency of about 1 in 20,000 (Jaenicke, 1982).

Volvox is a useful organism to demonstrate interdependence between cells in a multicellular organism. The multicellular nature of this eucaryotic alga is at an early stage of evolution and the individual

cells are recognisably related to the unicellular Chlamydomonas.

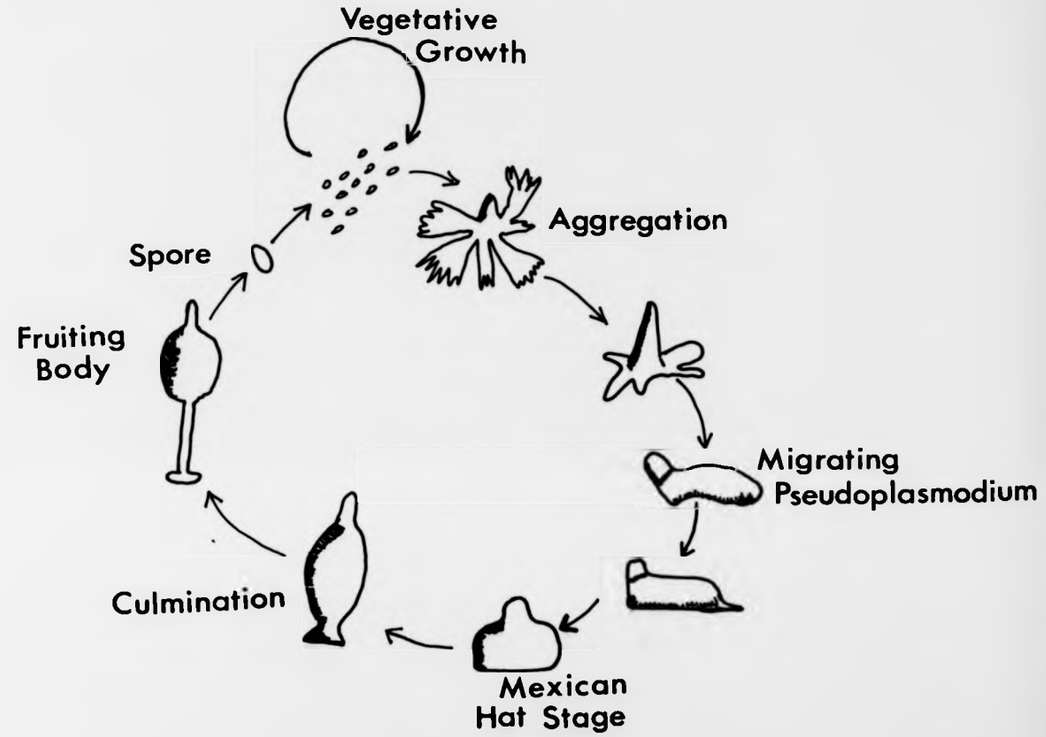
1.2(b)(v) Cellular Slime Moulds. The cellular slime moulds also show co-operation and interdependence between cells but in this case the individual cells resemble unicellular amoebae (Blumberg and Lodish, 1980a). Before discussing the slime moulds it is interesting to note that a free living soil amoeba Acanthamoeba castellani undergoes also cell differentiation during its life-cycle. Differentiation of vegetative cells to dormant cysts can be initiated in the presence of high cell densities or in conditions of starvation. The cellular differentiation involves the degradation of numerous cellular components and synthesis of new macromolecules (McMahon et al., 1980).

The slime moulds or Myxomycetes are found on decaying logs in damp woods and have a vegetative structure known as a plasmodium consisting of a multinucleate mass of cytoplasm which flows in an amoeboid fashion over the substrate while ingesting microbes. Fruiting occurs when the plasmodium migrates to a dry area and a complex fruiting body is formed bearing large numbers of uninucleate spores which germinate to produce amoeboid gametes which fuse to give zygotes that develop into a new plasmodium.

The slime moulds also include a small group including Dictyostelium which show far greater resemblances to unicellular amoebae. The vegetative phase consists of small uninucleate amoebas which multiply by binary fission. However thousands of these isolated amoebae are capable of aggregating and, without losing their cellular distinctness, form a fruiting body containing spores that germinate to produce individual amoebae. The life cycle of Dictyostelium discoideum consists of two mutually exclusive phases (see Figure 1.18; Blumberg and Lodish, 1980a). The vegetative phase includes growth, DNA synthesis, increase in cell

Figure 1.18 (Modified from Jacobsen and Lodish 1975)

Fruiting body formation in Dictyostelium discoideum.



mass and cell division and the organism exists as unicellular amoeboe feeding on soil bacteria. The development phase includes fruiting body construction and hence cell differentiation, pattern formation and morphogenesis. Development is initiated by the removal of nutrients and may be inhibited by the addition of nutrients up to the culmination stage. The cells aggregate to form a tissue-like assemblage of cells known as the grex or pseudoplasmodium. This passes through a number of stages starting with vertical elongation into a slug-like shape which lowers itself to the substratum and migrates. At the end of the migration the tip returns to a vertical position and fruiting body formation begins at this culmination stage. The cells at the tip develop into a stalk and as this increases in length the cells at the rear ascend it and finally differentiate into spores. The fruiting body exhibits simple pattern formation and morphogenesis which is size invariant with about 2 spores per stalk cell.

Epigenetic phenomena such as cell contact, positional information, and morphogenetic movements play an important part in embryogenesis in many organisms. Little is known however of the molecular mechanism by which these events influence the pattern of gene expression. Differentiation in D. discoideum is initiated by chemotaxis and cAMP signalling in the early stages of development resulting in the formation of loose aggregates of cells (Alton and Lodish, 1977c). At this stage cell interactions are needed to produce differentiation into spore and stalk cells. Synthesis of some regulated proteins is dependent upon continued cell-cell contact. A developmental axis is specified by a tip structure forming on the aggregates and positional information determines whether a cell differentiates to produce a spore or a stalk cell. In the culmination phase a series of morphogenetic movements takes place resulting in the final differentiation of spore and stalk

cells (Blumberg and Lodish, 1980a).

In Dictyostelium the regulation of protein synthesis is sensitive to the environment. In response to starvation and anoxia there is a rapid reduction in the initiation of protein synthesis and a reduction in the size and amount of polysomes (Cardelli and Dimond, 1981). Two dimensional gel electrophoresis shows that no new proteins are synthesized during the first 60 minutes of differentiation. The main change seen is the cessation of synthesis of five proteins and the reduction in synthesis of others. When cytoplasmic mRNA is isolated, by hybridisation to poly (U), from growing cells after 15 minutes development and translated in a wheat germ cell free system, proteins are synthesised which are not made in cells after 2 minutes development has occurred. This suggests that the changes in the pattern of protein synthesis occurring during the first hour of differentiation are due to regulation at the translational level (Alton and Lodish, 1977a). Two different types of RNA excess hybridisation techniques probing the complexity and diversity of the polyadenylated mRNA populations during differentiation showed that the only time when the initiation of a significant number of genes occurs is midway through the developmental programme (Blumberg and Lodish, 1980b). This is the point at which multicellularity is achieved just prior to differentiation into spore and stalk cells. About 3,000 genes produce RNA transcripts at this stage and represent about 30% of the polyA RNA with the remaining 70% being composed of 5,000 RNA species expressed throughout growth and early development. This pattern of gene expression shows parallels with that seen when an embryonal carcinoma cell differentiates to a committed myoblast cell line with the presence of 4-5,000 additional polyadenylated RNA species (Alton and Lodish, 1977b). This differentiation is also preceded by cell to cell contact as in

Dictyostelium.

1.2(b)(vi) Saccharomyces cerevisiae. I have already discussed some aspects of differentiation and morphogenesis in Mucor and Candida but the yeast whose cell cycle has been most studied is Saccharomyces cerevisiae (Hartwell, 1974). The yeast cell reproduces by budding and the bud grows in size through the cell cycle until division. A number of landmark events can be observed during the cell cycle such as bud emergence, DNA synthesis, nuclear division, and cell division (see Figure 1.19). The organism possesses a useful genetic system with cells of ploidy from haploid to octaploid allowing recessive mutations to be observed in haploids, analysed by complementation in diploids and gene dosage effects to be studied in polyploids. There are homologies between yeast cells and higher plant and animal cells in cell structure, chromosome replication and segregation and macromolecule synthesis (Hartwell, 1974). Using two-dimensional polyacrylamide gel electrophoresis it has been found that the histones H4, H2A and H2B are synthesized only in the late G1 and early S phases (Jorincz et al., 1982). There were also eight proteins whose rates of synthesis varied during the cell cycle and nine proteins which may have been regulated by periodic synthesis, modification and degradation. None of the cell-cycle regulated proteins now found is a major protein on two-dimensional gels and the 150 to 200 most abundant proteins are invariant in synthesis during the cycle (Jorincz et al., 1982). Earlier work had also shown that most protein synthesis is not periodic in the S. cerevisiae cell cycle with each of 550 major proteins synthesized in every fraction of the cell cycle (Elliott and McLaughlin, 1978). Ribosomal proteins were also found to be synthesized continuously through the cell cycle and did not show periodic variation (Elliott et

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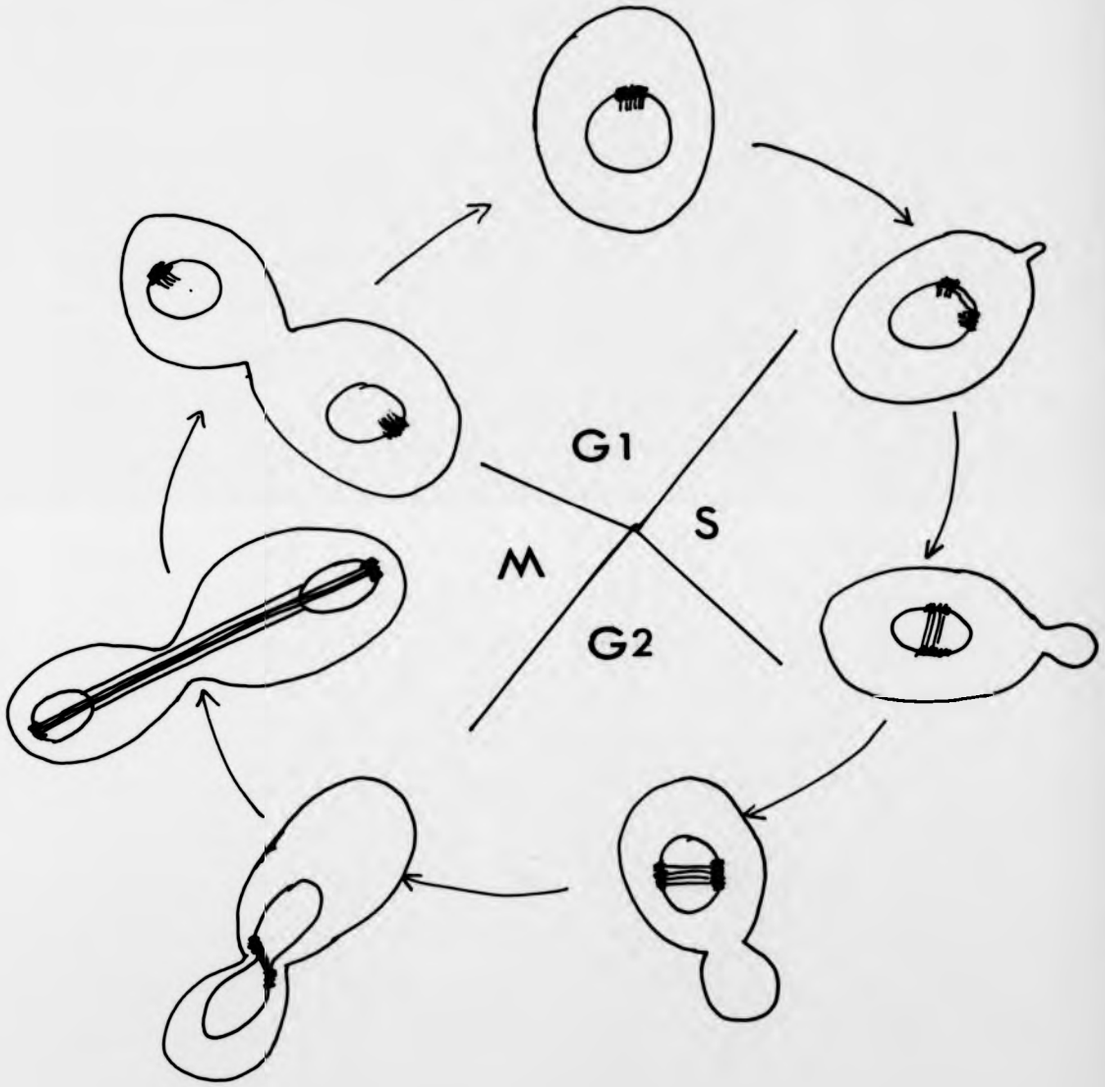
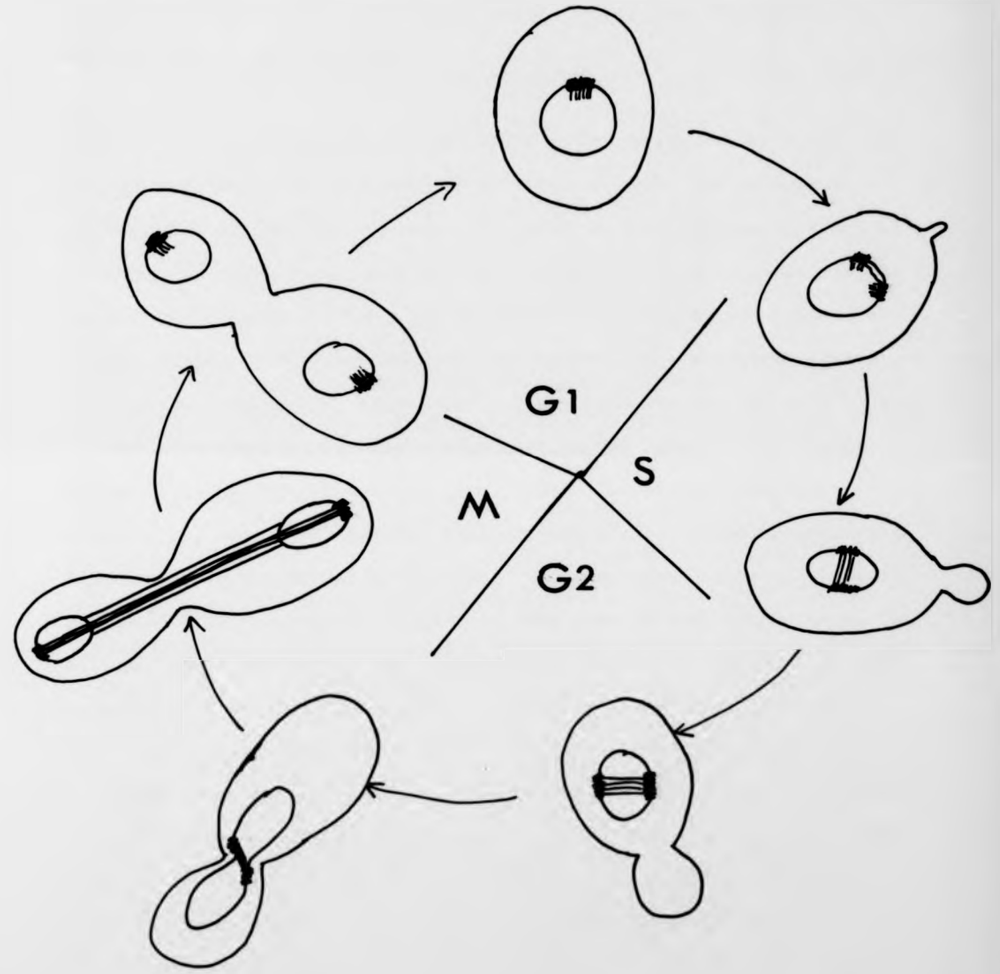


Figure 1.19 (Modified from Hartwell 1974)

Cell division cycle of Saccharomyces cerevisiae showing the landmark events of bud emergence and chromosome replication during the S phase, mitosis and cell division.



al., 1979). It appears that only a small number of less common proteins are synthesized periodically and that periodic synthesis, degradation, or modification is not a feature of the S. cerevisiae cell cycle (Elliott and McLaughlin, 1979).

1.2(b)(vii) Higher Animals. The study of DNA sequences by restriction and sequence analysis has produced information about the structural organisation of eukaryotic genes. In order to study gene expression at the level of individual genes one needs a system in which genes, in the form of purified cloned DNA, are faithfully transcribed and translated. Using a nuclear infection technique the oocytes of the African clawed toad Xenopus laevis have been used to transcribe DNA species such as the 5S rDNA from erythrocytes and cloned histone DNA from the sea-urchin Psammechinus milians (Probst et al., 1979). The histone DNA was accurately transcribed together with non-specific erroneous transcripts from the antisense strand of the DNA. The erroneous transcripts remain sequestered in the nucleus suggesting some form of copy-choice mechanism that excludes all but faithful transcripts from the cytoplasm. The DNA of eukaryotic cells is present as a complex nucleoprotein structure known as chromatin the composition of which may vary greatly with regard to proteins and RNA species complexed to the DNA according to the method of isolation employed (O'Malley et al., 1977). Non-histone DNA binding proteins have been implicated as potential candidates for regulating gene expression. Some of the evidence that suggests this includes:

(i) Non-histone proteins are present at increased levels in tissues active in RNA synthesis while histones are not.

(ii) Non-histone proteins show tissue specificity and DNA binding specificity.

(iii) Some of the non-histone proteins stimulate RNA synthesis in vitro.

(iv) The synthesis of certain classes of non-histone proteins is associated with the induction of gene activity.

How the non-histone proteins may act if they have a regulatory function is speculative (O'Malley et al., 1977). They may lower activation energy at the initiation sites for opening the DNA strands in forming a stable binary complex with RNA polymerase. They may also alter histone-DNA interactions to create a structural alteration and produce a transcriptionally active region (O'Malley et al., 1977).

1.2(b)(viii) Higher plants. If we now consider green plants, it is found that when etiolated pea seedlings are exposed to light for 24 hours then returned to darkness 38% of Chl a, 74% of Chl b and 84% of the light harvesting chlorophyll a/b protein that had accumulated in the light is broken down in darkness. In contrast the large and small subunits of ribulose 1,5 bisphosphate carboxylase continues to accumulate in darkness although at a slower rate (Bennett, 1981). Messenger RNA species encoding the light harvesting proteins can be detected in the polysomes of intermittently illuminated leaves and these messages are active in producing light harvesting protein precursors in a wheat germ "run off" system. Thus the inability of intermittently illuminated leaves to accumulate light harvesting proteins is not due to selective inhibition of translation but because they undergo rapid turnover in the absence of stabilisation by Chl a and Chl b which are not synthesised under dark conditions (Cuming and Bennett, 1981).

1.2(b)(ix) Diversity in eukaryotic life cycles. The range of complexity in eukaryotes resembles that seen for prokaryotes. There are in both cases unicellular organisms such as E. coli and Saccharomyces cerevisiae and systems of increasing complexity involving multicellular

organisms such as Rhodomicrobium vannielii and Volvox carteri. In the higher eukaryotes co-operation between cells, differentiation of cells, specialised cells, and even forms similar to unicellular amoebae such as macrophages are present complexed together in most organisms. In Dictyostelium and Volvox we see co-operation and co-ordination between cells and these organisms perhaps illustrate early stages in the evolution of the higher eukaryotes.

Some forms of cell expression such as the dimorphic cell types found in Mucor and Candida and the production of resting cells are not found in the higher eukaryotes and the study of these facets is likely to be of less relevance to the study of higher eukaryotic development than the primitive co-operation and co-ordination seen in Dictyostelium and Volvox.

1.2(b)(x) The cell cycle in eukaryotes and prokaryotes. The eukaryotic cell cycle may be divided into a number of phases (see Figure 1.20). DNA synthesis takes place in the S phase followed by a phase G2 without further DNA synthesis before mitosis and division occur. After division a further phase G1 without DNA synthesis occurs before the S phase begins again. Cells that are not destined for an early repeat of division are usually arrested at a G0 within G1. Cells arrested in G2 have however been identified.

This pattern of chromosome replication and division in eukaryotes is similar to that of prokaryotes described in section 1.2(a)(i). The phase of DNA synthesis C is followed by a phase D after termination before the cell divides. A variable phase I follows division and is directly analogous to the G1 plus G0 phases of the eukaryotic cell cycle (see Figure 1.3). In fact the eukaryotic nomenclature has been used in the cell cycles of some bacteria such as Caulobacter (see Figure 1.11;

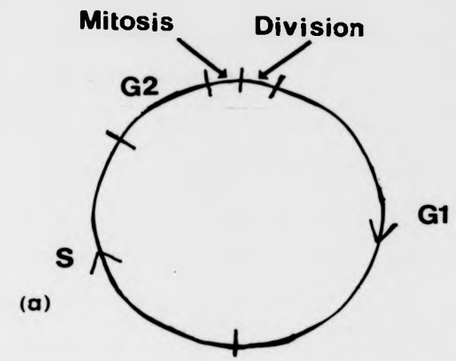
Figure 1.20

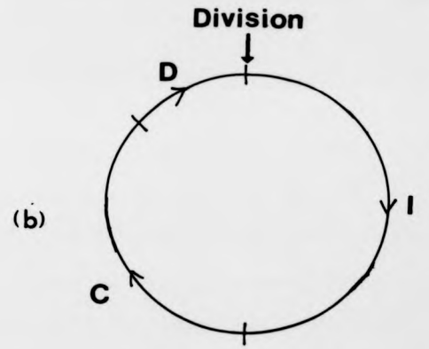
Comparison of cell cycle in eukaryotes and prokaryotes.

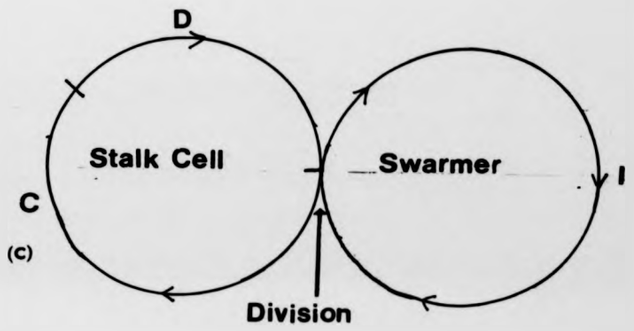
a) Cell cycle in eukaryotes.

b) Cell cycle in prokaryotes such as E. coli with a variable "I" period which may be of zero duration.

c) Cell cycle in budding bacteria where the swarmer cell requires a maturation or "I" period but the stalked cell can re-enter DNA synthesis immediately.







Osley and Newton, 1977). In budding bacteria the G1 or I phase represents the time needed for the maturation of a swarmer cell to produce a stalked reproductive cell and in the mother cell this phase is lacking and DNA synthesis can be initiated immediately.

In eukaryotes a cytoplasmic trigger for the initiation of cell division and DNA synthesis is suggested by nuclear transplantation experiments. When nuclei were removed from cells in G1 or G2 phases to cells in S phase, DNA replication occured. Conversely a nucleus transplanted from an S phase cell to a cell in a different phase stops DNA synthesis. When Tetrahymena is deprived of an essential amino acid in G1 phase replication never occurs suggesting a critical protein is synthesised at the G1-S boundary as removal of the amino acid in S phase does not prevent DNA synthesis.

In E. coli the initiation of DNA synthesis has been correlated with the synthesis of a membrane protein involved in DNA attachment to the origin of replication (Helmstetter et al., 1979).

The close correlation between the eukaryotic and prokaryotic cell cycles makes it possible that they are controlled in a similar way by inhibition or triggering of DNA synthesis. The budding bacteria are especially suitable for a study of the cell cycle because the swarmer cell alone exhibits an I or G1 phase and can be held in this phase by environmental factors.

1.3 Control of Gene Expression and Differentiation

An attractive hypothesis of differentiation is that it involves sequential switching on of a number of genes, with each new round of sequential protein synthesis being controlled by one of the products of

the previous round. If this were so the proteins synthesised during differentiation and development should alter in a characteristic fashion. However in many of the developmental systems which have been studied, including organisms such as E. coli and Saccharomyces cerevisiae, almost all proteins are made in every stage of the cell cycle. In other organisms with, in general, more complex development such as Caulobacter, Myxococcus, Dictyostelium, significant numbers of proteins are periodic in synthesis during the cell cycle. Even here the majority of proteins are synthesised throughout the cell-cycle or developmental sequence.

Development in higher eukaryotes follows highly ordered programmes of sequential gene expression as seen for example in the puffing of Drosophila polytene chromosomes (Ashburner, 1969) and silkmoth choriogenesis (Nadel and Kafatos, 1980).

The differential activation of bacterial genes is classically seen as being controlled by repressor proteins in the operator-promoter concept of Jacob and Monod (1961). In this model repressor proteins bind specifically to operators and prevent RNA polymerase from initiating transcription at the adjacent promoter. The repressor can interact with small molecules that provide signals from the environment. However a large number of control mechanisms can be envisaged in the control of gene expression and cell differentiation and there is evidence that the majority of them actually occur in one system or another. Briefly, the controls cover the field from gene dosage and DNA structure at one extreme, through transcriptional and translational controls of various types, to proteolysis and the direct control of the synthesis of supramolecular structures. The evidence for each of these control mechanisms is now considered.

1.3.1 Gene dosage

If a gene cannot supply sufficient mRNA or rRNA when being transcribed at the maximum rate then there will be an evolutionary selection pressure to increase the number of copies of that particular gene. In Escherichia coli the maximum rate of re-initiation at the ribosomal RNA gene promoter is about one per second. When E. coli is growing rapidly it can duplicate in about 20 minutes which would allow for the synthesis of about 1200 ribosomes worth of rRNA if there were only one gene for rRNA. The E. coli cell however contains about 10,000 ribosomes and in order to synthesise sufficient rRNA seven copies of the rRNA gene are found dispersed throughout the bacterial chromosome. The dispersal of multiple copies provides greater stability in the absence of selection pressure for multiple copies at low growth rates. Gene dosage as a form of control is a slow response type needing many generations to have its effect (Zubay, 1980).

1.3.2 Gene transposition

The regulation of gene expression by mechanisms involving site specific recombination is seen in the mating type system of the yeast Saccharomyces cerevisiae where the switch from "a" mating type to "α" mating type occurs when a replica of a silent "α" gene replaces an "a" gene at the mating type locus in a cassette model (Kushner et al., 1979; see Figure 1.21 for the life cycle of S. cerevisiae).

In mice the immunoglobulin polypeptide chains consist of an amino terminal variable V region and a constant C region encoded in embryonic cells in widely separated DNA segments. During differentiation of B lymphocytes the DNA segments are brought into proximity by site specific recombination enabling many different V regions to be combined with a single C region (Sakano et al., 1979).

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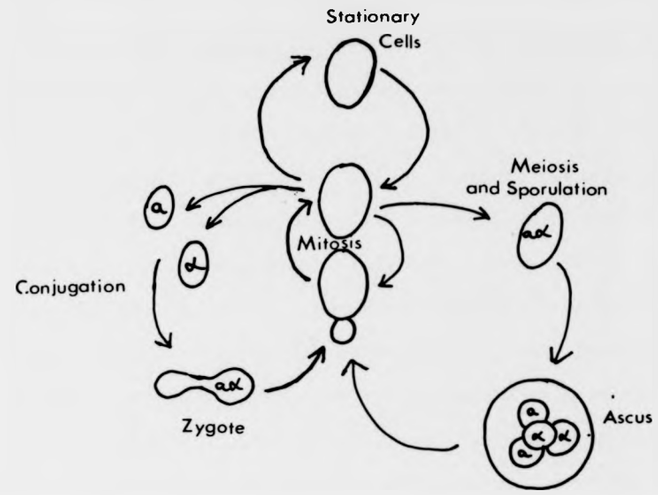
Figure 1.21 (Modified from Hartwell 1974)

Life cycle of Saccharomyces cerevisiae. Haploid cells may be "a" or "α" and diploid cells "aα", and all these types can undergo mitosis and enter stationary phase. Mitotic haploid cells may fuse with cells of the opposite mating type to create a diploid zygote which produces "aα" mitotic cells. The diploid cells can undergo meiosis and sporulation and the resulting "a" and "α" spores germinate when nutrients are replenished.



In Salmonella two genes, H1 and H2, code for the flagella monomer flagellin and these map in different regions of the genome (Silverman and Simon, 1980). Salmonella has the ability to switch between expression of the two flagellin structural genes and this presumably allows evasion of the host immune response. This is known as phase variation (Lederberg and Lino, 1956). Genetic and physical analysis of recombinant DNA molecules showed that inversion of 900 base pairs adjacent to the H2 operon controlled the expression of this gene. In one orientation H2 is "on" and in the opposite orientation H2 is "off". The 900 base pair invertible region that controls phase variation includes a gene which codes for a 19K protein that catalyses site-specific inversion (Silverman and Simon, 1980). When H2 is switched "on" H1 is not expressed because another gene linked to, and co-ordinately expressed with, H2 codes for a repressor to prevent expression of H1. When H2 is switched "off" H1 can be expressed. The frequency of phase variation is about 10^{-3} to 10^{-5} per cell per generation, and the switch of a population from one form to the other is completed by the selection pressure from the immune system of the host (Silverman and Simon, 1980). The advantage of these switching mechanisms is most pronounced in bacteria which may produce large populations clonally from a single ancestor during invasion of a host. The switching mechanism reduces the homogeneity of the clone and the effectiveness of the host's immunological system. Tn5-112, a deletion mutant of the kanamycin resistance transposon Tn5, can act as a model of an invertible element and switch on and off the beta- galactosidase gene in E. coli K12 (Berg, 1980). The deletion in Tn5-112 removes transcription termination signals and allows transcription initiated within the element to continue into nearby bacterial genes. Thus in one orientation the transposon stimulates distal gene expression while in

the other orientation polarity intervenes and gene expression is not stimulated. As Tn5- 112 contains terminal inverted repeats intramolecular recombination can invert the element.

In addition to the example found in Salmonella recombinational switches have been found in the phages Mu and P1 which determine which of two alternative host range specificities the progeny virus will exhibit (Berg, 1980).

Another example is provided by the gal operon in Escherichia coli where the 1400 base insertion element 1S2 can integrate near the start of the operon in either of two orientations (Zubay, 1980). In one orientation it prevents expression of the gal genes while in the other orientation it causes constitutive expression and it may be hypothesized that 1S2 contains a promoter.

1.3.3 DNA structural changes

Changes in the primary, secondary and tertiary structure of DNA can influence transcription of genes (Zubay, 1980). Primary structure changes include transient single strand cleavage which is known to encourage transcription in vitro and mature T5 phage DNA contains five single stranded breaks (Zubay, 1980). Base modifications are also primary structure changes and in T4 phage DNA hydroxymethylation of cytosine is an aid to late transcription (Zubay, 1980). Analogues of cytidine, such as 5 aza-cytidine, resistant to methylation induce changes in the phenotype of mouse embryo cells in culture which develop into striated muscle cells and adipocytes several days after exposure (Jones and Taylor, 1980). In vitro DNA methylation in the 5' region of the human gamma globin gene introduced into mouse L cells prevents transcription while methylation in the gamma globin structural regions does not (Busslinger et al., 1983). This suggests that methylation of

cytosine to 5 methylcytosine is involved in the control of gene expression and that DNA modification in general may be involved in development.

Secondary structure changes involve the transition from double stranded to a single stranded structure. In some viruses such as T4 bacteriophage some late genes are only transcribed where DNA is being replicated and because this involves the transient appearance of single-stranded DNA it is possible that some promoters are only recognized in the single stranded form (Zubay, 1980). It appears that the DNA in prokaryotic chromosomes is coiled in nucleosome like structures, although in vivo only part of the DNA is arranged in this way (Pettijohn, 1982). The DNA in the isolated nucleoid is negatively supercoiled and the chromosome segregated into distinct domains of supercoiling such that the torsional tensions can be maintained independently in each domain (Pettijohn, 1982). Regulation at this level could provide a basis for co-ordinate control of operons in each domain. It is known that the introduction of negative supercoils into DNA stimulates the in vitro template activity. Possibly the explanation for this is that the formation of rapid start complexes partially unwinds the double helix introducing positive supercoils. If the DNA is negatively supercoiled the energy for unwinding the double helix can be supplied by unwinding the supercoils (Zubay, 1980). It is also possible that RNA polymerase can more easily enter negatively supercoiled DNA. The degree of supercoiling of DNA can differentially activate promoters and there are sites on the the DNA separate from promoters where topoisomerases can act to produce this differential gene expression (Smith, 1981). Topoisomerase I, also known as omega protein, relaxes negatively supercoiled DNA.Mutations in the topoisomerase I gene (supX) restore expression of promoter mutations in the leu operon of Salmonella

typhimurium (Smith, 1981). A possible interpretation of this is that some promoters are active only when the DNA is fully negatively supercoiled by DNA gyrase. In the absence of topoisomerase DNA remains negatively supercoiled and the mutant leu operon can be expressed. Inhibitors of DNA gyrase such as nalidixic acid inhibit transcription from some promoters but not others while topoisomerase I can also prevent expression of some promoters by relaxing the DNA. Some promoters can be turned on either by a specific activator protein or by supercoiling. For example the lac operon is activated by a CAP-cAMP complex and is inactivated by DNA gyrase inhibitors but activated by topoisomerase I mutations (Smith, 1981). It is possible that the CAP-cAMP complex activates promoters by unwinding DNA locally while supercoiling achieves this over a whole domain, with DNA gyrase and topoisomerase I acting at particular sites to control the expression of all the genes in a domain. DNA gyrase synthesis may itself be controlled by the degree of supercoiling in E. coli in a homeostatic mechanism with relaxed DNA being a good template for in vitro DNA gyrase synthesis and supercoiled DNA a poor template (Menzel and Gellert, 1983).

DNA supercoiling and its effect on gene expression may extend to the eukaryotes. The template surface of DNA is buried within the double helix and must be exposed during transcription by unpairing bases. Just such an unpairing is induced by negatively supercoiling the DNA. As a result, supercoiled duplexes are cleaved by single strand specific nucleases and bind single strand specific chemicals. If HeLa cells are lysed in 2M sodium chloride nucleoids are released which contain nuclear DNA packaged within a flexible cage of RNA and protein (Akrigg and Cook, 1980). The DNA behaves as if it were unbroken and supercoiled. Topoisomerases which decrease and increase supercoiling, i.e. untwisting

enzyme from rat liver and DNA gyrase from Escherichia coli, can be used to twist and untwist the nucleoid DNA and alter the degree of exposure of bases. The supertwisting produced by the action of DNA gyrase dramatically increases transcription by RNA polymerase II from wheat germ. In vivo eukaryotic DNA is folded around histone cores to form nucleosomes and the isolated complex contains no free energy that can be released by the untwisting enzyme (Akrigg and Cook, 1980). It is thought that this makes it unlikely that there is a eukaryotic counterpart to the bacterial DNA gyrase. It is however an attractive hypothesis to suppose that a eukaryotic gyrase acts to expose the template surface, stimulate transcription and so determine the activity of genes. Some evidence for the existence of a eukaryotic DNA gyrase in HeLa cells is provided by the observation that the DNA of cells grown in the presence of novobiocin, a specific inhibitor of the bacterial gyrase, is less negatively supercoiled than if not treated (Akrigg and Cook, 1980). This form of topological gene control has received support recently (Lilley, 1983). One important observation is that the reactivation of stored yeast mating type gene copies on plasmids which are normally silent is accompanied by alteration of the plasmid topological state.

1.3.4. DNA dependent RNA polymerase modification and replacement

In Escherichia coli only a single species of DNA dependent RNA polymerase is to be found. The core enzyme consists of three different subunits, alpha, beta and beta-prime. Another subunit, the sigma factor, is needed to give full activity on native DNA. The core enzyme plus the sigma factor, known as the holoenzyme, has the structure $\alpha_2\beta\beta'\sigma$ and the molecular ratios of the subunits are about 37,000, 155,000, 156,000 and 90,000 respectively. The molecular ratio (M_r) of

the holoenzyme is thus about 500,000 and is one of the most complex enzymes in the bacterial cell (Lathe, 1978). However mitochondrial RNA polymerase is a single polypeptide of M_r 60,000. The complexity of the bacterial enzyme probably reflects the need for transcriptional regulation and RNA polymerase may be an allosteric enzyme that can differentiate between different promoters. Some control factors may operate by converting the enzyme from one form to another. In eukaryotes there are three structurally distinct enzymes. Polymerase A is found in the nucleolus and synthesizes rRNA. Polymerase B is found in the nucleoplasm and synthesizes heteronuclear (hn) RNA while the third, polymerase C synthesizes 5S and tRNA. The eukaryotic polymerases are all heteromultimers with similar but more complex structures to the bacterial enzyme. Like the prokaryotic enzyme the eukaryotic polymerases probably show allosteric specificity changes with each type having a restricted initiation range (Travers, 1976).

The entire replacement of an RNA polymerase is seen in some bacteriophage infections. When phage T7 infects *E. coli* one of the early genes transcribed by the host polymerase codes for a viral polymerase which transcribes the remainder of the viral genome while the host polymerase is inhibited by proteins encoded by the early T7 genes (Zubay, 1980).

Apart from complete replacement the existing structure of the RNA polymerase could be directly modified so that the specificity for different promoters is changed. The structural changes could be brought about by subunit replacement or subunit modification. Several lytic phages, for example T5, SP01, produce proteins *in vivo* which bind to the host polymerase and alter the transcriptional specificity (Zubay, 1980; Doi, 1977). During the formation of virions and endospores in *Bacillus subtilis* sequential gene expression is controlled by regulatory sigma

factor proteins that modify the promoter recognition specificity of the RNA polymerase. In B. subtilis the core enzyme can interact with at least six proteins to achieve a number of different promoter recognition specificities (Doi, 1977). The growth of phage SP01 in B. subtilis involves expression in a temporal sequence controlled at the level of promoter recognition (Doi, 1977). The early genes are expressed immediately after infection and are recognised by the unaltered RNA polymerase containing sigma^{55}. After 5 minutes the middle genes are expressed by the action of an early protein, gene product 28 or sigma^{SP28}, which binds to the core enzyme and displaces sigma^{55}. In a similar way late gene transcription is activated by gene products 33 and 34 or sigma$^{SP33-34}$ acting synergistically. In this way the temporal programme of gene expression can be explained by a cascade of phage encoded sigma factors. In order to investigate the promoter sequences recognised by RNA polymerases with different sigma factors, regions of DNA protected from DNAase I nicking by DNA polymerase were investigated (Doi, 1977). These regions were 60-70 base pairs long and extended from 40-50 bases preceding the start of RNA synthesis to about 20 bases past the start point (i.e. from -40 to -50 to position +20). The binding sites recognised by E. coli RNA polymerase containing the sigma factor showed 2 regions of homology. From position -30 to position -35 called the -35 region the most common sequence is 5'TTGACA while from position -12 to -7 called the Pribnow box the most common sequence is 5'TATAAT (Doi, 1977). The sequence of the SP01 early gene promoter recognised by B. subtilis RNA polymerase differs from this by only one nucleotide in both the -35 region and the Pribnow box and both polymerases transcribe SP01 early genes. In contrast two SP01 middle gene promoters show distinctive sequences in both regions. The sequence of the -35 region is 5'AGGAGA while the Pribnow box region contains 5'TTTATTT (Losick and

Pero, 1981; Talkington and Pero, 1979).

Endospore formation in B. subtilis involves five temporally defined classes of gene expression. The developmental programme is initiated by a class of seven regulatory genes known as the spo0 genes which are probably vegetative genes with regulatory products that induce the expression of early sporulation genes in response to nutrient limitation. As with the phage development discussed previously, sporulation can be correlated with the appearance of regulatory proteins that modify the specificity of the RNA polymerase. The factor sigma^{37} is present in vegetative cells together with sigma^{55} and sigma^{37} directs the transcription of two cloned sporulation genes that are not recognised by RNA polymerase containing sigma^{55}. About 1 hour after the start of sporogenesis sigma^{55} and sigma^{37} are replaced by sigma^{29} the synthesis of which is a sporulation specific event. The role of multiple sigma factors is not confined to sporulation as another factor sigma^{28} is found in vegetative cells as is sigma^{37} and could control other gene pathways such as genetic competence.

In both sporulation and phage development sigma^{55} is replaced by newly synthesized factors. Sporulating cells are known to produce a product that dissociates sigma^{55} from RNA polymerase (Losick and Pero, 1981). The appearance of this sigma dissociating factor is under developmental control and mutations in certain spo0 regulatory genes prevent the removal of sigma^{55}. Promoter specificity is probably determined by direct interaction of the sigma factor acting as part of the holoenzyme with specific sequences in the promoter region. The factors sigma^{37} and sigma^{28} recognise sequences distinct from those recognised by sigma^{55} in both the -35 region and in the -10 region or Pribnow box.

	-35 region	-10 region
sigma^{55}	5' TTGACA	5' TATAAT
sigma^{37}	5' GG-T-AAA	5' TATTGTTT
sigma^{28}	5' CTAAA	5' CCGATAT

Both the sporulation and phage examples suggest that each sigma factor dictates the recognition of specific sequences at both the -35 and -10 regions during the formation of the RNA polymerase-promoter complex. One can envisage two models of how sigma factors produce specificity. The sigma factor could change the conformation of the enzyme so as to enable it to recognise the -35 and -10 regions. However there are at least 6 species of sigma factor and 4 types of sequence at -35 so it seems unlikely that the enzyme could adopt so many different conformations. The second model involves direct contact between the sigma factor and the -35 and -10 regions either simulataneously or sequentially during RNA polymerase-promoter complex formation (Losick and Pero, 1981; Haldenwang and Losick, 1979). Ultra-violet radiation forms covalent cross-links between *E. coli* RNA polymerase and a *lac UV5* promoter substituted with bromouracil in place of thymine. It is then found that the sigma subunit is linked to base -3 and the beta is linked to base +3. The Pribnow box is in the region -6 to -12 so this region may form specific cross links with the sigma factor (Simpson, 1979).

B. subtilis has a number of sigma factors both in vegetative and sporulating cells and there are more phage specific factors. These factors may recognise different spectra of promoters to provide specificity of gene expression. In *E. coli* on the other hand there is only one sigma factor and specificity of gene expression must be achieved by other means (Doi, 1982). There is some evidence for the existence of different conformational structures of the *E. coli* RNA

polymerase. Sedimentation of RNA polymerase on glycerol gradients shows a separation of molecules into functionally distinct populations that differ in template preference as shown by transcription of cloned fragments of DNA. The differences in sedimentation position may reflect conformational differences in the polymerase molecule. The nucleotides ppGpp and ppApp induce opposing effects on the sedimentation characteristics of the enzyme. The nucleotides may alter the position of an equilibrium between different structural conformations of the enzyme and thereby alter its promoter specificity (Travers et al., 1980a). Ribosomal RNA is synthesized most efficiently by molecules in the trailing shoulder of the peak of RNA polymerase on a glycerol gradient. Preparations of the initiation factors IF2a and IF2b both stimulate the in vitro synthesis of rRNA which may be contrasted with IF3 which has no effect on rRNA synthesis and fMet-tRNA which inhibits rRNA synthesis (Travers et al., 1980b). The effect of IF2 could be explained by assuming that it converts RNA polymerase conformations to those having a lower sedimentation coefficient. This would be compatible with a role in which accumulation of free initiation factors would indicate that ribosomes were fully utilised in protein synthesis and that the cell needed rRNA synthesis.

In Lactobacillus curvatus three different forms of Rna polymerase have been found, the core enzyme E, a holoenzyme complexed with factor Y, Ey and a holenzyme complexed with a sigma factor E.sigma. The Y factor, M_r 84K, displaces the sigma factor, M_r 44K, from free E.sigma so that an excess of factor Y, the only free species of polymerase should be Ey. The factor Y prevents E.sigma from binding to non-promoter sites from which it cannot initiate transcription and enhances specific activity by a factor of two. Ey and sigma form complexes with promoters

which are converted to stable preinitiation complexes with the release of Y. After the start of elongation sigma is released from the nascent tertiary complex (Gierl et al., 1982).

1.3.5 Regulation of initiation of transcription by control factors

The initiation site on the DNA for transcription contains a rapid start binding site for RNA polymerase and in many cases binding sites for regulatory proteins which either inhibit or facilitate polymerase binding. Negative control involves a repressor which excludes the RNA polymerase from promoter sites. In the case of the lac operon in the absence of lactose a lac specific repressor protein binds to the lac promoter and physically excludes the polymerase from binding to that site. Expression of the lac operon requires an antirepressor such as lactose or a derivative of lactose, or a synthetic antirepressor such as isopropyl-beta-D-thiogalactoside (Zubay, 1980). The antirepressor binds to the repressor causing an allosteric transition which lowers affinity for the promoter. Positive control involves an activator which binds adjacent to the promoter site to stimulate transcription. In the lac operon the absence of an active repressor is not a sufficient condition for the full expression of the operon. An activator is also needed which is a complex between the catabolite gene activator protein (CAP, CRP) and cAMP. Positive control also occurs in the arabinose utilization ara operon, where ara C protein and CAP protein are apoactivators needing L-arabinose and cAMP respectively in order to function. The presence of glucose can reduce expression of operons such as lac and ara needing a CAP-cAMP complex for initation (Zubay, 1980). This catabolite repression of operons is mediated by positive control

involving cAMP and the catabolite gene activator protein. This complex can also exert negative control inhibiting the expression of other genes. Genes that are negatively controlled in this way include glutamine synthetase and outer membrane protein III (Mallick and Herrlich, 1979). In fact, studies with two-dimensional polyacrylamide gel electrophoresis show that cAMP strongly represses nearly as many genes as it regulates positively. Negative control by cAMP depends on the presence of the catabolite gene activator protein as does positive control but it is established at a lower cAMP concentration and may involve the synthesis of a superrepressor protein stimulated by cAMP.

A regulatory protein has been implicated in the control of heat shock proteins in Escherichia coli (Neidhart and Vanbogelen, 1981). A group of nine proteins in E. coli K12 vary with growth temperature and are abundant above 40°C. The proteins are recognised by their high rates of synthesis during a ten minute period following a shift up in temperature. This produces a 20 fold stimulation in their synthesis which is abolished by a mutation elsewhere in the chromosome. Since this possible control gene maps at a location distant from the genes it controls and since this function is blocked by an amber mutation it is probable that the gene codes for a positive regulatory protein that stimulates expression of the high temperature proteins above 40°C.

In eukaryotes the 5S RNA of cytoplasmic ribosomes is synthesized separately from the other ribosomal RNAs using RNA polymerase III. In immature oocytes from Xenopus laevis and X. borealis where large numbers of 5S rRNA molecules are produced on highly reiterated genes prior to ribosome formation a factor of M_r 40,000 is essential to 5S rRNA transcription (Garrett and Noller, 1982). This factor binds to nucleotides 45 to 96 of the 5S rRNA gene and is assumed to direct the

action of RNA polymerase III. The factor can constitute about 15% of the total oocyte protein and is identical to the protein associated with 5S RNA in immature oocytes. This suggests a mechanism of control by feedback inhibition. When 5S rRNA is in excess it could compete for the factor halting the initiation of transcription which occurs at the stage of oocyte maturation and ovulation. The factor is degraded prior to ribosome assembly which occurs after transcription of the amplified genes of the large ribosomal RNAs. The dual binding property of the factor to DNA and 5S rRNA suggests an analogy with the mechanism of translational control of ribosomal protein synthesis when proteins which bind strongly to rRNA in the ribosome feedback when in excess and bind to a structurally related site on their mRNA thereby inhibiting translation (Garrett and Noller, 1982).

The nucleotide guanosine 5' diphosphate 3' diphosphate or ppGpp accumulates in bacteria in response to both energy starvation and amino acid starvation and is in general accompanied by a reduction of protein and RNA synthesis to basal levels (Lagosky and Chang, 1980). Mutants lacking ppGpp have been isolated showing that the nucleotide is not essential for growth but the ability of such a mutant to undergo growth transitions is impaired (Travers, 1980). Correlating with increased ppGpp levels is the cessation of rRNA synthesis indicating a change in transcriptional selectivity (Richter et al., 1979). The presence and immediate effects of regulatory nucleotides may be short lived but they may have long term effects. The accumulation of ppGpp in Escherichia coli is accompanied by the synthesis of two proteins B56.5 and stringent starvation protein (Travers, 1980). The latter binds to RNA polymerase and alters its properties in a similar manner to ppGpp itself. Thus the nucleotide induces the synthesis of proteins which stabilise the effects

that it itself produces. There may be another example of this in Bacillus subtilis where the 37,000 M_r polymerase binding protein which appears in the initial stages of sporulation could stabilise the effects of the transient perturbation of the adenine and guanine nucleotide pools. In bacteria the major role of guanine nucleotides is in translation which generates GDP from GTP so ppGpp and other nucleotides could be derived from a regulatory system using GDP and GTP to couple transcription and translation (Travers, 1980).

The stringent response in bacteria co-ordinates the synthesis of rRNA and tRNA with the availability of amino acids through the synthesis of ppGpp and pppGpp. ppGpp also inhibits the in vitro synthesis of ribosomal protein L12 (Chu et al., 1976). In Escherichia coli and Bacillus subtilis stringent factor, an ATP.GTP pyrophosphate transferase, is produced when uncharged tRNA is bound to ribosomes in amino acid starvation (Richter et al., 1979). In some bacteria such as Bacillus stearothermophilus and B. brevis the synthesis of stringent factor does not depend on ribosomal activation. The nucleotide ppGpp is degraded to GDP by the removal of pyrophosphate and this ppGpp cycle is present in a number of unrelated bacteria including Thiobacillus ferrooxidans and Thermus thermophilus (Richter et al., 1979) in addition to those mentioned above. The metabolic pathway is:

$$\text{GDP} + \text{ATP} \xrightarrow[\text{AMP}]{\text{stringent factor}} \text{ppGpp} \xrightarrow[\text{PPi}]{\text{ppGpp ase}} \text{GDP}$$

Stringent factor is coded for by the relA locus and relA mutants appear to retain a basal level of ppGpp synthesis which is independent of stringent factor, and in wild type bacteria is mutually exclusive with

stringent factor mediated synthesis (Logosky and Change, 1980). Relaxed bacteria in contrast to the wild type greatly reduce their ppGpp synthesis in response to amino acid starvation and this is accompanied by increases in RNA synthesis. In contrast glucose or carbon source starvation in relaxed bacteria causes an increase in ppGpp synthesis and a reduction in rRNA synthesis. This inverse relationship between ppGpp synthesis and RNA synthesis in relaxed bacteria and stringent wild type bacteria suggests that ppGpp is the control element of rRNA synthesis and that the basal level of ppGpp synthesis is involved in the control of rRNA synthesis during balanced growth (Richter et al., 1979; Lagosky and Chang, 1980).

In addition to acting as a negative regulator in stable RNA synthesis ppGpp may also act as a positive effector. Expression of the his operon in Salmonella typhimurium in an in vitro transcription-translation system is dependent upon the addition of ppGpp (Stephens et al., 1975). In Escherichia coli both the lac and trp operons are stimulated by 200 μM ppGpp and the nucleotide may be a general transcriptional effector interacting with RNA polymerase to exert its influence in a gene-specific manner (Reiness et al., 1975).

A type of stringent response has been observed in the cyanobacterium Anacystis nidulans but in this case a shift down by reduction in light intensity produced a reduction in stable RNA synthesis and increased the concentrations of ppGpp and pppGpp (Mann et al., 1975). After shift down there is also a rapid rise and fall in GTP levels. Stable RNA was not synthesized for some time after ppGpp and pppGpp had returned to basal levels suggesting that another mechanism, perhaps the synthesis of a protein, makes the response more long-lived.

This method of control, i.e. the stringent response, may also be

present in eukaryotes as the so called "Magic Spot" which accumulates during differentiation in Dictyostelium discoideum in response to starvation has the same Rf as ppGpp on polyethyleneimine cellulose chromatography (Klein, 1974). The addition of formycin B to aggregates in a late stage of fruiting body formation causes regression to the migrating slug stage which will fruit later if not further interfered with. Formycin B is an inhibitor of guanosine metabolism and may exert its morphogenetic effect by interfering with guanosine polyphosphate synthesis or breakdown (Cohen and Sussmann, 1975). Guanosine polyphosphates have also been observed to be synthesized in spinach chloroplast and rat liver mitochondrial preparations with exogenous GDP or GTP. However in these cases the regulatory role of the nucleotides may be restricted to the organelles, rather than in a eukaryotic system as such (Horvath et al., 1975).

Another nucleotide, cyclic guanosine 3'-5' monophosphate, has been implicated as a cell cycle regulator. Transient increases in the intracellular level of cGMP have been seen with a periodicity of one generation time in Escherichia coli and Bacillus licheniformis. Cyclic GMP could be involved in the regulation of the cell cycle and may relate to control exerted by ppGpp and pppGpp (Cook et al., 1980).

The stringent response is an interaction of components of the translation machinery with transcription and the products inhibited are primarily parts of the translation system such as rRNA, tRNA, r-protein and translation factor mRNAs. Other forms of translation-transcription interaction can be envisaged. Formyl-methionyl-tRNAfMet will bind strongly and specifically to RNA polymerase in E. coli and stimulate transcription. This could represent a cellular tool to couple the rate of mRNA transcription with mRNA translation and allow the cell to exert

general metabolite control on transcription and translation in a concerted fashion. The association of nascent RNA with ribosomes provides a further coupling between transcription and translation. The activity of RNA polymerase is inhibited by the RNA product and ribosomal binding to the 5' terminus or the interaction between fMet-tRNAfMet and mRNA could relieve this (Pongs and Ulbrich, 1976).

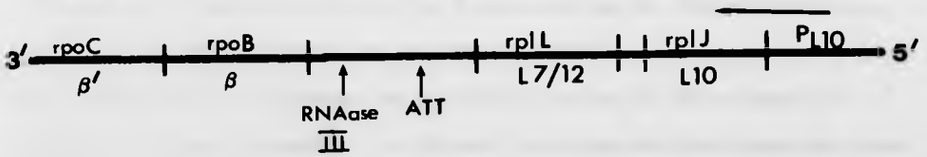
1.3.6 Regulation of termination of transcription by control factors

If the termination of transcription were provisional it could be used to regulate transcription of those genes after the signal. In the E. coli temperate bacteriophage lambda a phage specified N gene product associates with the host polymerase and alters the termination but not the initiation specificity (Doi, 1977). This allows readthrough to phage developmental genes located after the termination signal. That this involves the regulation of rho factor interaction with the RNA polymerase is shown by the fact that host strains with an altered rho factor can support the growth of N deficient phage mutants.

Another form of termination control occurs when a nascent transcript is terminated before the structural genes are reached. In the trp operon an attenuation site 141 nucleotides from the initiation site stops 90% of transcripts when optimal (Zubay, 1980). Low levels of $Trp\text{-}tRNA^{Trp}$ allows readthrough of the attenuator while tryptophan concentration controls the promoter. Thus the proportion of transcripts that elongate beyond the attenuator depends on the cells need for tryptophan. In a system such as this with two metabolic switches the amplification factor from "full off" to "full on" is the product of that from each switch. Whether RNA polymerase terminates or reads through at

the attenuator is determined by the alternative secondary structures in the leader transcript. The transcript of the trp leader region contains a coding sequence for a 14 residue polypeptide which has two adjacent tryptophan residues. Under tryptophan starvation conditions when the cell is deficient in Trp-tRNA^{Trp} the ribosome translating the leader transcript stalls at the pair of trp codons. This promotes the formation of a particular secondary structure in the transcript that allows transcription to proceed beyond the attenuator. When there is an adequate supply of tryptophan the entire leader peptide sequence can be synthesized, an alternative secondary structure forms in the mRNA transcript, and termination is favoured (Das et al., 1982).

The close metabolic ties which exist between transcription and translation are reflected in the organisation of the genes which code for the core proteins, beta and beta', of RNA polymerase and two ribosomal proteins, L7/12 and L10, in E. coli (Lang-Yang and Zubay, 1981). The genes for the polymerase subunits rpoB and rpoC and the genes for the two ribosomal proteins rplL and rplJ are part of a single operon. This operon contains a single strong promoter at P_{L10} and the promoter proximal gene rplJ is separated by 69 bases from the second gene rplL which is separated by 325 bases from rpoB.



Only about 20% of mRNA transcripts initiated at P_{L10} transcribe through the entire operon. The remainder are terminated at an attenuator site 69 bases downstream from the rplL gene. A further 131 bases downstream there is a cleavage site recognised by RNAase III. All transcripts that

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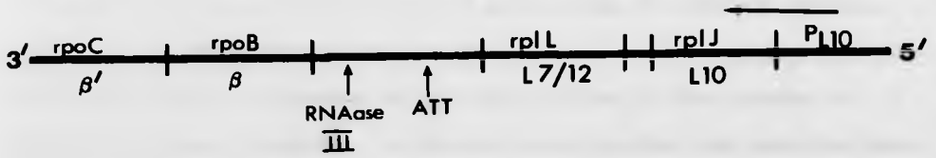

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go beyond the attenuator are cleaved at the RNAase III site so that the rpoBC part of the message can function as an independent unit during subsequent translation. The clustering of genes for RNA polymerase and ribosomal proteins within the same operon allows simultaneous regulation of the synthesis of proteins required for transcription and translation. Cells usually have a greater demand for ribosomes than RNA polymerase subunits and the four gene products in the operon L10, L7/12, beta and beta' are produced in molar ratios of 1:4:0.2:0.2 during exponential growth, resulting from the attentuaton of 80% of transcripts. Superimposed on this control there is feedback inhibition of translation of their mRNAs by both the RNA polymerase subunits and the ribosomal proteins. Amber mutations in the rpoB gene specifying the beta subunit coupled with conditional amber suppressors has been used to restrict synthesis of the RNA polymerase core. This stimulates transcription of the beta and beta' genes suggesting that feedback inhibition also acts at the transcriptional level perhaps at the attenuator (Little and Dennis, 1980).

Escherichia coli may also have the capacity to co-ordinately regulate the synthesis of DNA and RNA by controlling initiation of DNA synthesis. The rpoD gene for the sigma subunit of RNA polymerase is situated in an operon with the dna G gene coding for the DNA primase. Initiation of DNA synthesis by the DNA polymerase III holoenzyme during DNA replication is dependent on the prior action of the primase to synthesise primer fragments. A plasmid carrying the dnaG gene has been constructed to amplify the intracellular primase levels 100 fold. Transcription directed by the lambda PL promoter on the plasmid, pRLM56, usually terminates at an attentuation site 30 bases upstream from the dna G gene. However if the RNA polymerase is first able to transcribe

across a Bgl III/Hind III *E. coli* DNA fragment in the plasmid then it is able to proceed past the attentuator more efficiently. This fragment may code for a bacterial factor that can modify the RNA polymerase so that it is able to transcribe past the attentuator more efficiently and transcribe the *dnaG* gene (Wold *et al.*, 1982).

1.3.7 Translational control mediated by secondary structure of mRNA

Prokaryotes and eukaryotes have evolved different ways of recognising their mRNAs. Prokaryotic mRNAs are polycistronic and initiation of translation can take place at internal sites on the message. On the other hand eukaryotic messages are functionally monocistronic and initiation is generally limited to the 5'-proximal initiation codons. A free 5' terminus seems to be indispensable for mRNA to interact with eukaryotic ribosomes since circularizing the mRNA prevents interaction with eukaryotic but not prokaryotic ribosomes. Prokaryotic ribosomes are also able to recognise changes in the secondary structure of mRNAs while eukaryotic ribosomes cannot. During initiation complex formation prokaryotic *E. coli* ribosomes bind to only one site on native prokaryotic phage f2 RNA or eukaryotic BMV RNA3. After irreversible unfolding of either RNA several AUG/GUG codons can be recognised by prokaryotic ribosomes and this produces polysomes. This would appear to be a property of the prokaryotic ribosome as the same result is obtained with both prokaryotic and eukaryotic messages. With wheat germ ribosomes phage f2 RNA and BMV RNA4 form only monosomes when the mRNA is irreversibly unfolded as in the case with native RNA. It seems that eukaryotic ribosomes cannot bind to internal AUG codons exposed by unfolding the mRNA (Zagarska *et al.*, 1982). mRNA selection

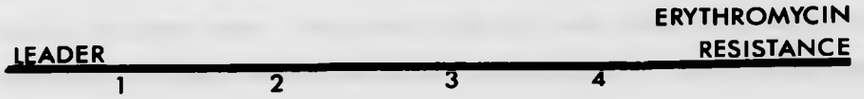
as a mechanism of gene regulation is suggested by the observation that lambda morphogenetic proteins vary by a factor of one thousand while the mRNAs vary only by a factor of two in concentration. There is complementarity between the 3' end of the 16S ribosomal RNA and sequences 5' to the AUG initiation codon. The 16S RNA sequence 5' ACCUCC 3' is postulated to bind to a polypurine rich Shine and Dalgarno region 5' to the initiation codon (Backendorf et al., 1980). Many initiation regions can be folded into secondary structures which may be favourable or unfavourable for translational initiation. The secondary mRNA structure may fix a proper spatial array of the Shine and Dalgarno region and the initiation codon. For example, in the phage T4 gene 38 the following structure can be constructed:

```
          UC
         U  G
          CG
          GC
          GC
          GU
          AU
          GC
5'  UAAGGAUAAAUAUG  3'
```

Shine and Dalgarno Initiation codon

In contrast an unfavourable structure may bury these regions and slow or prevent initiation.

A similar model has been proposed to account for the induction of erythromycin resistance in Staphylococcus aureus. Two hairpins formed from domains 1 plus 2 and 3 plus 4 buries initiation codon in secondary structure (Gold et al., 1981).



Low levels of erythromycin bind to ribosomes and halts them in the leader sequence so that domain 1 is within the ribosome domains allowing 2 and 3 to form a hairpin thus freeing domain 4 and the initiation codon. This system could be arranged to respond to a protein binding to mRNA so freeing another region for translational initiation.

Translation of mRNA may require that part of its secondary structure is altered, transiently or permanently, by specific proteins. In prokaryotes ribosomal protein S1 from E. coli and an analogous protein from Caulobacter crescentus, which are required for the binding of mRNA to ribosomes, disrupt the secondary structure of various RNA and DNA molecules (Marvil et al., 1980). RNA binding proteins may also repress translation, for example, in the regulation of synthesis in RNA coliphages by phage specific coat and replicase proteins. In eukaryotes many embryonic systems contain an inactive protein rich form of stored mRNA. For example in encysted gastrulae of the brine shrimp Artemia salina there is a single stranded nucleic acid binding protein which is a potent inhibitor of protein synthesis and this may play a role in the regulation of translation. The nucleic acid binding protein forms regularly spaced beadlike structures about 20 nm in diameter along the length of the nucleic acid molecule as seen by electron microscopy (Marvil et al., 1980).

1.3.8 Translational control mediated by the primary sequence of mRNA

The primary sequence of individual mRNA species may determine the efficiency of their translation both by frequency of initiation and choice of codons used. The codon usage has been found to be markedly different in highly expressed genes in comparison to those coding for

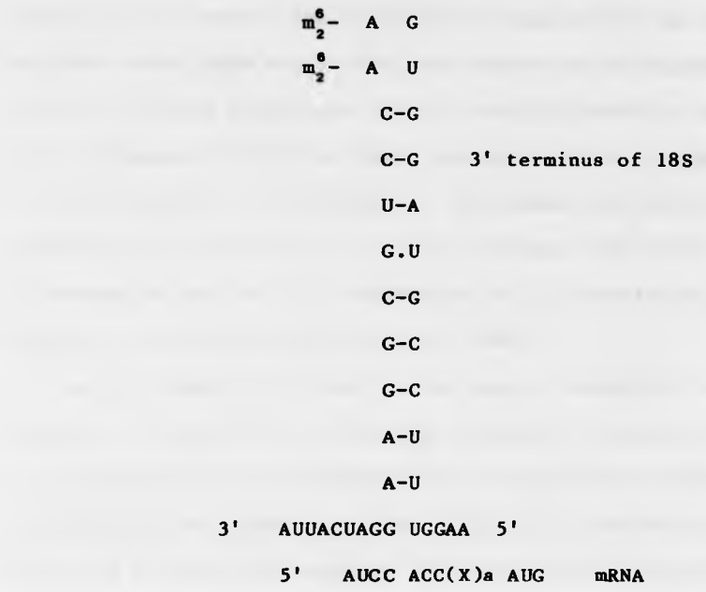
rare proteins such as repressors (Grosjean and Fiers, 1982). Variation in the rate of elongation in translation can be a means of regulating gene expression both directly by slowing or accelerating the rate of protein synthesis and indirectly by altering the three dimensional structure of the mRNA when the progress of the ribosome is disturbed. If a specific aminoacylated tRNA is present in limiting concentrations the overall rate of protein synthesis is approximately determined by the relative rate of reading past an individual codon requiring that particular aminoacylated tRNA raised to the power of how many times that codon appears in the mRNA, suggesting that a proportion of ribosomes fall off the message at each "hungry" codon. However under starvation conditions for a particular amino acid reading through a "hungry" codon occurs at a surprisingly high level presumably due to misreading of the codon (Goldman, 1982). There have been associations of different specific tRNA concentrations with differentiated cell types and the specialized products that such cells synthesize. It has also been suggested that the frequency of a codon in genes correlates with the abundance in the cell of the tRNA isoacceptor species specific for that codon. So variations in codon usage could control differential synthesis of protein and changes in specific tRNAs in differentiated cells could control changes in the spectrum of proteins synthesized.

In *E. coli* the *rpoD* gene for the sigma subunit of RNA polymerase is situated in an operon with the *dnaG* gene for the primase needed to synthesize the primer for initiation of DNA synthesis and this arrangement may reflect co-ordinate regulation of DNA and RNA synthesis (Wold *et al.*, 1982). The intracellular concentration of sigma is 20x higher than the primase and this is possibly due to a weak ribosome binding site and an unusually large number of rare codons in the *dnaG*

gene that hinders its translation.

Codon usage in the E. coli lipoprotein mRNA is unusual. There are 15 amino acids in the prolipoprotein and 10 of these are coded for by only the most abundant codon, i.e. only the major isoaccepting tRNA species is used. The remaining five amino acids need only two isoaccepting tRNA species each. The usage of only major isoaccepting tRNA species is a very efficient way to translate genes of abundant proteins (Nakamura et al., 1980).

Eukaryotic mRNAs do not contain the clearly defined Shine and Dalgarno sequence which is thought to act as a ribosome binding site on prokaryotic mRNAs. This conserved purine rich sequence on the 5' leader sequence is thought to base pair with the sequence ACCUCCU at the 3' end of the 16S rRNA. A novel interaction has been proposed between the 5' untranslated region of eukaryotic mRNAs and non-contiguous sequences of the 18S rRNA which has at its 3' end a heavily conserved hairpin which is stabilised when the message's 5' leader sequence base pairs with conserved nucleotides flanking the hairpin. Sequences close to the required 5' AUCCACC 3', which base pairs with these conserved nucleotides, occur commonly in eukaryotic mRNAs immediately upstream from the initiation codon.



The interaction may have a role in the events which lead up to the initiation of protein synthesis, and the sequence and variations of it may control the rate and efficiency of translation from a particular initiation codon (Sargan et al., 1982).

1.3.9 Translational repressors including self regulation

The rates of synthesis of most ribosomal proteins in exponentially growing cells of Escherichia coli are identical and respond co-ordinately to changes in growth rates (Jinks-Robertson and Nomura, 1982). An explanation for this is that ribosomal protein synthesis and ribosome assembly are coupled so that when ribosomal protein synthesis exceeds that needed for ribosome assembly the excess ribosomal proteins can interact with their mRNAs and block translation. Specific ribosomal proteins such as S4, S7, S8, L1, L4 and L10 can act as translational

repressors and inhibit the synthesis of some or all the ribosomal proteins whose genes are in the same operon as the repressor. Ribosomal protein L1 exerts autogenous control and also inhibits the synthesis of L11. The genes for both of these proteins are on a single operon with L11 most proximal to the promoter. Ribosomes that initiate protein synthesis at the beginning of the L11 message very likely translate the L1 message as well so that inhibition of L11 translation would also inhibit L1 translation (Brot et al., 1981).

As previously discussed, in the operon coding for the ribosomal proteins L10 and L7/12 and the RNA polymerase subunits beta and beta' the transcription of the message for the polymerase subunits is controlled by an attenuator. In addition to this the ribosomal proteins L7/12 and L10 act as autogenous inhibitors for the translation of their respective mRNAs, and RNA polymerase appears to act as a selective inhibitor for the translation of the mRNA for beta and beta' (Lang-Yang and Zubay, 1981; Fiil et al., 1980).

In a DNA dependent protein synthesizing system for the transcription and translation of Escherichia coli genes carried by transducing lambda phages the products included the ribosomal protein S20 and isoleucyl tRNA synthetase. The production of S20 is specifically stimulated by the addition of 16S rRNA but not by 5S or 23S rRNA. Addition of 16S rRNA to separate transcription and translation systems showed that it is the translation of the S20 mRNA which is enhanced. This is explained by feedback inhibition by protein S20 at the translational level relieved by the incorporation of S20 into ribosomal assembly by binding to the 16S rRNA. In contrast the synthesis of the isoleucyl tRNA synthetase in vitro is not influenced by the addition of purified enzyme nor by the addition of anti-serum to it,

so appears not to be autogenously controlled (Wirth et al., 1981).

In eukaryotic systems a translational inhibitor has been purified from wheat germ extracts which inhibits protein synthesis in rabbit reticulocyte lysates (Ranu, 1980) The inhibitor contains a cAMP independent protein kinase activity that phosphorylates the 38,000 M_r subunit of rabbit reticulocyte eIF-2 Alpha. Protein synthesis in intact reticulocyte lysates is dependent on the presence of heme which binds to a protein kinase diminishing its activity and thus its ability to inhibit the initiation of protein synthesis. In addition to the heme regulated translational inhibitor a dsRNA activated protein kinase has also been isolated from rabbit reticulocytes. All three protein kinases are cAMP independent and phosphorylate the same 38,000 M_r subunit of eIF-2 Alpha raising the possibility that phosphorylation-dephosphorylation of eIF-2 Alpha may be an important mechanism in eukaryotic protein synthesis (Leroux and London, 1982).

A small RNA molecule has been implicated in translational inhibition (Bag et al., 1980). Deproteinised mRNAs, isolated from ribonucleoprotein complexes present in the cytoplasm of eukaryotic cells, are translatable in a cell-free system. Non-polysomal mRNA-protein complexes are in some cases translatable but in other cases fail to promote protein synthesis in a cell-free system and also inhibit the translation of other mRNAs. An example found in chicken embryonic muscle consists of a 10 to 15S ribonucleoprotein which lacks mRNA but contains a 4.4S RNA molecule. Both the 4.4S RNA and the RNP inhibit the translation of other mRNAs in a cell free system.

1.3.10 Post-transcriptional processing of mRNA

In an Escherichia coli lysogenic for lambda spc2 transducing phage

an extra copy of ribosomal protein genes is carried on the phage chromosome. In the merodiploid the synthesis of ribosomal protein mRNA appears to be gene-dosage dependent but the amount of functional message is independent of the gene dosage. The authors suggest that, in addition to feedback inhibition by ribosomal proteins, a specific nuclease recognises the r-protein-mRNA complex as substrate and inactivates excess mRNA. This is also put forward as a possible explanation of the RNA polymerase beta and beta' subunit regulation (Fallon et al., 1979).

The E. coli enzyme RNAase III was the first specific RNAase to be discovered and purified. RNA metabolism includes a step in which mature RNAs are cleaved from larger primary transcripts. RNAase III cleaves dsRNA structures and it is thought that mRNA precursors contain small dsRNA regions separating the individual mature messenger segments. The cleavage of dsRNA is optimal at monovalent cation concentrations of about 0.1 M and under these conditions the enzyme is a dimer. Under low salt concentrations of 0.01 M the enzyme is monomeric and unpaired RNA is cleaved (Robertson, 1982).

A new and surprising form of post-transcriptional regulation has been discovered involving RNAase III. In this mechanism, termed retroregulation, expression is regulated from a site promoter-distal to the structural sequence of the mRNA. The RNA in this region has a hyphenated dyad symmetry so that it can adopt the structure with two stems and two loops characteristic of the RNA substrate of RNAase III. This allows RNAase III to process mRNA containing the regulatory site with the loss of messenger activity (Gottesman et al., 1982).

In eukaryotes post transcriptional processing may involve 3' polyadenylation, 5' capping, or removal of introns and these may have a

regulatory role. The 5' cap of inverted 7-methylguanosine facilitates the process of initiation by accelerating the rate at which mRNA binds the initiation complex (Hellmann et al., 1982).

Introns, not as yet known in prokaryotes, are possible regions of regulation. The specific removal of introns can give rise to mRNAs of various structures with different combinations of structural coding sequence.

The 3' polyadenylylation of mRNA is almost universal among eukaryotes but there are few reports of its presence in prokaryotes (Kerjan and Szulmajster, 1980). A population of polyadenylylated RNA appears during sporulation in Bacillus subtilis while little or no poly(A)mRNA is found in vegetative cells or in the stationary phase cells of an asporogenic mutants blocked at the zero stage of sporulation. The poly(A) mRNA may play a role in the regulation of gene expression during sporulation and it may be that the greater stability of poly(A) mRNAs is needed in the resting spore.

1.3.11 Control of gene expression by proteolysis

There are three ways in which proteolysis could affect gene expression. It is possible that gene expression could be altered by selective proteolysis of repressor or gene activator proteins. By using ^{35}S methionine labelling of proteins and immunoprecipitation of the bacteriophage lambda repressor, the degradation of the repressor following induction with mitomycin C has been demonstrated. Degradation in vitro requires Mg^{2+} ions and ATP as well as an active recA product (Maurizi and Switzer, 1980).

In Bacillus thuringiensis during sporulation the sigma subunit is converted from a form with M_r 58,000 to a M_r 50,000 form $(sigma)_m$

(Maurizi and Switzer, 1980). A radiolabel chase experiment shows that this is formed by modification of the vegetative sigma rather than new synthesis. In this same system a modified beta' subunit $(beta)_m$ M_r 130,000) appears at the same time as the vegetative beta' (M_r 150,000) disappears. An intracellular protease has been purified that catalyses the production of a polymerase containing $(beta)_m$ *in vitro*. However the protease only converts the polymerase, known as form II, from sporulating cells and does not attack the vegetative enzyme. Form II polymerase has lost the vegetative sigma factor but is otherwise indistinguishable from the vegetative enzyme. Changes in transcriptional specificity appear to be correlated with the loss of the vegetative sigma factor rather than the modification of the beta' subunit.

If the selection of mRNA species during the transition from vegetative growth to sporulation were governed by initiation factors of differing specificity, proteolysis could alter translational control by modification or degradation of vegetative initiation factors. Also if ribosomal proteins underwent specific proteolytic modification during sporulation the modified ribosomes could be altered in their ability to translate specific mRNA species or to synthesize highly phosphorylated nucleotides. There is little evidence for these speculations however.

There may be a role for proteolysis during the germination of spores. Free amino acids are released from germinating spores of *Bacillus megaterium* from the degradation of certain spore proteins which have M_r 6-12,000 and high isoelectric points of about 9.5 (Maurizi and Switzer, 1980). These proteins comprise 15-20% of total spore proteins and are degraded in the first 20 to 40 minutes of germination. Seven protein species account for almost all the protein that is degraded. It

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is possible that these proteins only provide a storage function for amino acids needed on germination but they also bind to DNA and raise its melting point so they could be involved in the spores resistance to radiation and high temperatures. It is also possible that the proteins are involved in the repression of vegetative genes during sporulation.

In contrast to mammalian organisms where virtually every protein is continuously degraded and resynthesized, in Escherichia coli the bulk of proteins are not catabolized to any extent. Three proteins have been found in E. coli cells which undergo a turnover process analogous to that in mammalian cells by two dimensional polyacrylamide gel electrophoresis and a double label technique with ^{3}H and ^{14}C leucine to measure changes in the radioactivity of isolated proteins (Larrabee et al., 1980). The changes found indicate a first-order breakdown. Apart from these three proteins the bulk of proteins in the soluble fractions and membrane fraction are found at levels proportional to the rate of synthesis and these levels decrease only as a result of cell division. The significance of the three proteins that are catabolized remains unknown.

Other authors have found that E. coli rapidly degrades proteins with abnormal configurations by an energy dependent process stimulated by ATP. The degradation of a nonsense fragment of beta-galactosidase ceases after several minutes in vitro unless ATP is added (Murakami et al., 1979). The lon(capR) gene product of E. coli K-12 has been identified as a 94,000 M_r polypeptide with an ATP dependent protease activity. One protein is present in the mutant capR strain that is absent from the wild-type capR^{+} strain and is proteolysed when pure capR^{+} protease is added to capR extract. This protein of M_r 11,000, pI approx. 4.5 is only found in the capR extract when cell division is

inhibited, and is either a substrate of the capR$^+$ protease or part of a cascade initiated by the protease. It may function in a cellular pathway which affects cell division as its appearance is associated with inhibition of cell division (Schoemaker et al., 1982).

1.3.12 Control of the synthesis of supramolecular strucutres

A simple model for pattern formation of the reproductive cells within the Volvox spheroid has been proposed and a glycoprotein implicated in the control of differentiation (Wieland, 1982). This is a sulphated surface glycoprotein of M_r 185,000 known as SSG-185 which turns over with a half-life of about 20 minutes. In vegetatively growing embryos the level of the glycoprotein declines during the divisions from the 4 cell stage to the 16 to 32 cell stage which is the last symmetrical cell division and where SSG-185 is at a minimum. In the next cleavage 16 out of the 32 cells divide unsymmetrically so that 16 out of 64 cells are committed to differentiate into reproductive cells and only the 48 somatic initial cells continue to divide. It may be hypothesized that SSG-185 is involved in cell to cell contact and that it suppresses unequal cell cleavage or induces somatic cell development. During successive cleavages the number of contacts increases exponentially, consuming SSG-185, and thus exhausting the pool at the 16 cell stage so that in the next division 16 cells are produced lacking SSG-185 on their surfaces (see figure 1.22). Differentiation cleavage is no longer inhibited in this subclass of 16 cells so that 16 gonidial initials are produced and then the suppression of unequal cleavage is restored by a rise in the level of SSG-185 after the unequal cleavage. The same phenomenon is observed during the sexual development in Volvox. In female strains the differentiation cleavage step is

Figure 1.22 (Modified from Wenzl and Sumper 1981)

Two dimensional model of a 16 cell Volvox embryo and transformation to a sphere by folding without steric hindrance. The cells labelled with a black dot have no SSG-185 containing contacts and should undergo unequal cleavage resulting in 8 gonidial initials arranged in two rectangles turned by 45° against each other in the anterior half of the embryo. This is the observed positioning in these Volvox embryos which undergo unequal cleavage at the 16 cell stage which occurs under poor growth conditions.

shifted to the 64 cell stage with a minimum level of SSG-185 at the 32 cell stage. During male reproduction the differentiating cleavage is yet more delayed to the 128 or 256 cell stage depending on species, the final division is asymmetric and leads to equal numbers of somatic and sperm producing cells. In the male case the minimum level of SSG-185 again occurs at the last cleavage before differentiation. Sexual reproduction in Volvox involves delaying the differentiating cleavage and this may involve control over the levels of the surface glycoprotein SSG-185.

In the E. coli cell cycle described in Section 1.2(a)(i) one model for the co-ordination of cell division with chromosome replication is that the physical presence of a chromosome or a chromosome-membrane attachment site prevents the formation of a septum (Tang and Helmstetter, 1980). This model suggests that chromosome replication and cell division follow separate pathways and that replication of a specific chromosomal region such as the terminus is not a mandatory requirement for the initiation and completion of the processes leading to cell division such as perhaps by the synthesis of a "division protein".

Once chromosome replication is complete the inhibition of septum formation is relieved, septum construction can proceed, and the cell enters the 20 minute D period. It is probable that the protein subunits needed to construct the septum are synthesized before the D period and construction proceeds when inhibition is removed. A heat labile protein required for division accumulates during the cell cycle reaching maximum concentration shortly before the division. Heat shock to 45°C delays division increasingly with cell age and division is delayed by 30 minutes late in the cycle suggesting that cell division timing depends

on building up a critical amount of the heat labile protein which may be a septum protein needed to complete the septum when segregation of the replicating chromosome occurs and cell division is unblocked (Smith and Pardee, 1970). A 50,000 M_r protein increases in E. coli membrane fractions when cells are prevented from dividing by use of mutants, inhibitors, or nutritional deficiencies. This protein may be the heat labile septum protein needed to complete the septum (Pardee, 1974). Similar results have been found by other authors using temperature sensitive mutants and a heat shock to 42°C which set back division by 55 to 60 minutes (Pedro et al., 1975). In this case resistance to heat shock was achieved 20 minutes before division at the start of the D period so this may not be a structural septum protein. Division was also found to be resistant to nalidixic acid and chloramphenicol in the D period so DNA and protein synthesis are not required in the final stages.

1.3.13 Summary

Briefly summarising the various methods by which gene expression and differentiation may be controlled it can be seen that almost every conceivable control level has been used in one species or another. It is no doubt of great advantage to organisms to have the ability to control macromolecular synthesis at a number of sites but it makes control of gene expression a complex problem to study and comparisons between different systems become hazardous. A large number of different explanations must be borne in mind when attempting to explain changes seen during the differentiation of any cell type.

1.4 The Use of Rhodomicrobium vannielii Swarmers as a Differentiation System

This study is concerned with the differentiation of the Rhodomicrobium vannielii swarmer cell to produce a mature reproductive cell and specifically with the changes in the pattern of protein synthesis seen during this differentiation. How are these changes controlled? Which of the many mechanisms for controlling gene expression outlined in Section 1.3 are involved in the differentiation of the swarmer cell?

These questions can only be answered by experiment but is the differentiation of swarmer cells a good system for the study of the regulation of gene expression? In Section 1.1 five criteria were quoted as being exhibited by the perfect system for the study of differentiation (Clark, 1971). How does swarmer cell differentiation measure up to these criterion in comparison to other much studied systems such as the E. coli cell cycle and Bacillus sporulation? The first criterion is that of simple and well defined morphological changes which can be synchronised. Stalk development and bud formation are easily observable microscopic characters and large numbers of swarmer cells can be obtained by selective filtration (see Section 2.5). In the cell cycle of E. coli the only morphogenetic event is cell division by binary fission and available selective synchronisation methods give only low yields.

The second criterion is growth in a chemically defined medium and in common with most bacteria that have been studied R. vannielii meets this. Most eukaryotes require complex growth media which complicates the study of biochemical changes associated with morphogenesis.

Clark's third criterion is that the system should be easy to work

with metabolically without major problems in making cell free extracts or isolating enzymes. No problems are encountered here with R. vannielii and it is also easy to introduce radiolabels into biological macromolecules such as protein, RNA, DNA and polysaccharides.

The fourth criterion is that the morphogenetic cycle should be subject to nutritional control and in this respect the control of swarmer differentiation by light intensity is a major advantage. In E. coli only the growth rate is subject to nutritional control while the control of sporulation in Bacillus probably by nutrient deprivation at the end of the exponential phase is not so convenient experimentally as the synchronous differentiation of swarmers when illuminated. Although R. vannielii cannot be grown without morphogenetic changes occurring as can for instance Arthrobacter or Chlorogloea but, as will be shown, swarmers maintained under a dark regime can take up ^{35}S L Methionine and incorporate it into proteins while in an inhibited state as far as differentiation is concerned. This allows proteins synthesised during the differentiation of the swarmer cell to be compared with those synthesised in the inhibited state by methods such as two-dimensional electrophoresis. In some bacteria such as E. coli there are no changes in the pattern of proteins synthesised through the cell cycle (Lutkenhaus et al., 1979). In the endospore formation of Bacillus species there are sporulation specific changes in protein synthesis implying differential gene expression. The existence of a second nucleoid in the developing forespore is an added complication in any postulated method of transcriptional control but protein turnover appears to be restricted to the mother cell compartment after forespore engulfment (Ellar et al., 1975).

Clark's fifth criterion, that cells should have a genetic system

that can be studied by the conventional methods with stable mutants and a system for the exchange of genetic information, has scarcely begun in R. vannielii. Few mutants have been discovered and exchange of genetic material has not been convincingly demonstrated. This contrasts with the well developed genetics of E. coli and Bacillus spp. The differentiation of swarmer cells is obligatory so temperature sensitive mutations are needed to provide blocks at different stages as is possible with Bacillus sporulation.

The genetic problems should not prove insuperable and with several other big advantages the differentiation of swarmer cells proves to be a good system for the study of gene expression during prokaryotic differentiaton.

2. METHODS

2.1 Strain

Rhodomicrobium vannielii Rm5 (Whittenbury and Dow, 1977) was used in this study.

2.2 Media

R. vannielii was grown on pyruvate-malate (PM) medium (Whittenbury and Dow, 1977) which contained per litre:

0.5 g Ammonium chloride (NH_4Cl)

0.4 g Magnesium sulphate ($MgSO_4.7H_2O$)

0.4 g Sodium chloride (NaCl)

0.05 g Calcium chloride ($CaCl_2.2H_2O$)

1.5 g Sodium pyruvate

1.5 g Sodium hydrogen malate.

The pH of the solution was adjusted to 6.8-6.9 with potassium hydroxide (KOH). For solid media 1.5% (w/v) Difco bacto-agar was added. The medium was autoclaved at 121°C for 15 minutes. After cooling 5% (v/v) sterile phosphate buffer was added aseptically.

0.1 M Phosphate buffer contained:

Sodium dihydrogen phosphate $NaH_2PO_4.2H_2O$ 7.8 g per litre.

Disodium hydrogen phosphate Na_2HPO_4 7.1 g per litre.

pH 6.8

2.3 Growth

R. vannielii was grown in 250 ml, B19 conical flasks sealed with rubber suba seals. Routinely a 1 ml inoculum was used in 100 ml of medium. Each flask was flushed with oxygen free nitrogen for 15 minutes through sterile syringe needles inserted in the suba seals, then incubated in a shaking water bath at 30°C and with an incident light intensity of approximately 1800 lux provided by tungsten lamps.

On a larger scale *R. vannielii* was grown in 5 litre, 10 litre and 20 litre flat-bottomed vessels (244/1350 - Baird and Tatlock) sealed with suba seals and flushed with oxygen free nitrogen for 30 minutes. These vessels were stirred with magnetic stirrers and incubated in a warm room at 30°C with an incident light intensity of 1800 lux.

2.4 Maintenance of Cultures

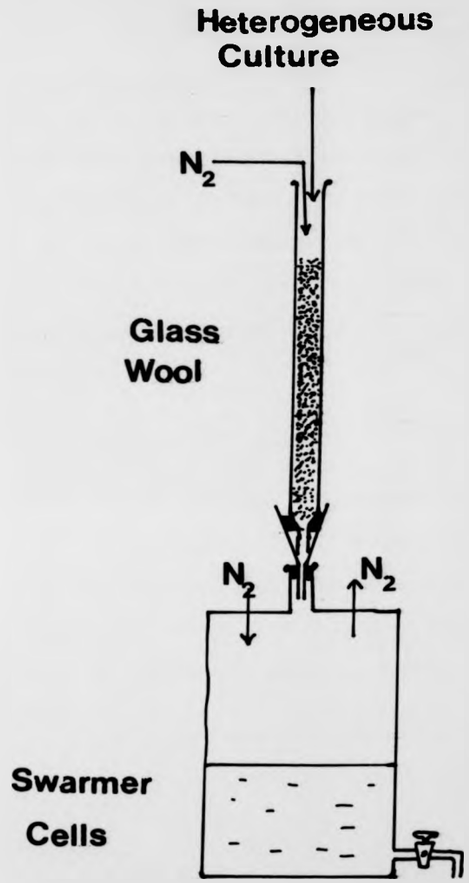
Culture purity was checked by phase contrast microscopy and purified if necessary by isolating individual colonies on PM agar plates. Cultures were maintained by transferring 0.5 ml samples of exponential cultures to sterile Eppendorf microcentrifuge tubes, freezing in liquid nitrogen and storing at -80°C.

2.5 Synchronisation of Swarmer Cells

Swarmer cells were obtained free from multicellular arrays by passing a late exponential phase culture of optical density (A_{540}. 2.0 to 3.0 through a sterile glass wool column (see Figure 2.1). The column was first washed with 1 litre of sterile distilled water and flushed continuously with oxygen free nitrogen. Multicellular arrays and

Figure 2.1 (Modified from Whittenbury and Dow 1977)

Synchronisation of swarmer cells using a glass wool column.



stalked cells were retained on the glass wool while the swarmer cells passed through and were collected in an aluminium foil covered vessel. Using columns of various dimensions synchronised homogeneous cultures from 500 ml up to 20 litres could be achieved. The homogeneity of the synchronized cells was checked using phase contrast microscopy and Coulter Counter cell volume distribution analysis. On removal of the aluminium foil from the culture vessel and incubation at 30°C and 2,000 lux the swarmer cells initiate differentiation into stalk cells with a high degree of synchrony as judged by microscopy and Coulter Counter cell volume distribution analysis.

2.6 Light Microscopy

Phase contrast microscopy was performed using an Olympus microscope fitted with an Olympus PM6 camera unit and phase contrast optics. Photomicrographs on Kodak Panatomic X film were developed in Kodak D19 developer at 20°C for 3 minutes and fixed with Kodafix. Prints were made using Kodak Veribrom paper.

2.7 Optical Density of Cultures

The optical density of cultures were measured at 540 nm (A_{540}) in a Pye-Unicam SP500 Spectrophotometer.

2.8 Size Distribution by Coulter Counter Analysis

All counts and size distribution analysis were performed on a Model ZBI Coulter Counter fitted with a Coulter Channelyzer C1000. Earlier

results were printed on an XY recorder while later results were fed in to a PET computer model CBM 8032, stored on a floppy disc using disc drive model CBM 8050 and printed when necessary. Average particle counts were taken as the mean of five determinations, using a 30 μm orifice tube. Latex particles were used as calibration standards.

2.9 Cell Carbon, Total Carbon and Inorganic Carbon Analysis

Carbon analysis was performed on a Beckman Total Organic Carbon Analyser Model 915-B using glucose and sodium carbonate as standards for total and inorganic carbon concentrations respectively. Cell carbon was determined by estimating total carbon before and after removal of cells from the culture by centrifugation.

2.10 Incorporation of L^{35}S Methionine into Cell Proteins

L ^{35}S Methionine was obtained from the Radiochemical Centre, Amersham, and was of specific activity >600 Ci mmol^{-1}. L ^{3}H Methionine from the same source was of specific activity 5-15 Ci mmol^{-1}. Radiolabel was added to complex and swarmer cultures usually at the rate of 1 μCi ml^{-1} for varying lengths of times and further incorporation prevented by the addition of unlabelled L methionine to 1 mM. Where necessary incorporation of radiolabel was slowed by the addition of unlabelled methionine, 2-5 μM being found sufficient. Cells were collected by centrifugation, drop frozen in liquid nitrogen and stored at -20^{o}C until required. Before lysis cells were washed (centrifugation in a microcentrifuge) with sonication buffer (O'Farrell, 1975) three times. The cells were lysed by sonication for 15 seconds every minute

for 10 minutes using microcentrifuge tubes immersed in a methanol-ice freezing mixture. Cell debris was removed by centrifugation at 10,000 g for 20 minutes and cell lysates stored at -20°C until required.

2.11 Determination of Radiolabel in Cell Lysates and Unbroken Cells

Samples were added to 2 ml of 5% (w/v) ice-cold trichloracetic acid and kept for 30 minutes at 4°C. The samples were then filtered through GFC glass filter papers and washed twice with 5% (w/v) trichloracetic acid, once with ethanol and once with a mixture of equal parts of ethanol and ether. The filters were put in a scintillation vial and dried at 60°C. Radioactivity on the filters was estimated after addition of 3 ml of Triton-Toluene PPO scintillation fluid in a Packard Tri-Carb scintillation counter. ^{35}S was counted with a gain settling of 13% with a counting efficiency of 80% and ^{3}H with a gain setting of 70% and a counting efficiency of 40%.

2.12 Determination of Protein Concentration

Protein concentrations were determined using the Folin-Phenol reagent with crystalline bovine serum albumin used as standard (Lowry et al., 1951). Briefly the method was as follows:

Solutions were :

A 5% (w/v) sodium carbonate (Na_2CO_3) solution

B 0.5% (w/v) cupric sulphate ($CuSO_4.5H_2O$) in 1% (w/v) sodium potassium tartrate [$NaK(CHOHCOO)_2$]

C 2 ml of B was added to 50 ml of A (made fresh daily)

D Folin-Ciocalteau phenol reagent diluted to 1.0 N acidity.

E 1 N sodium hydroxide (NaOH) solution

The procedure was:

To the sample was added 0.5 ml of 1 N sodium hydroxide solution, 2.5 ml of solution C, it was then allowed to stand for 10 minutes. Solution D (0.5 ml) was then added and allowed to stand for 30 minutes. The optical density was then measured against a reagent blank with distilled water at 650 nm in 1 cm cuvettes.

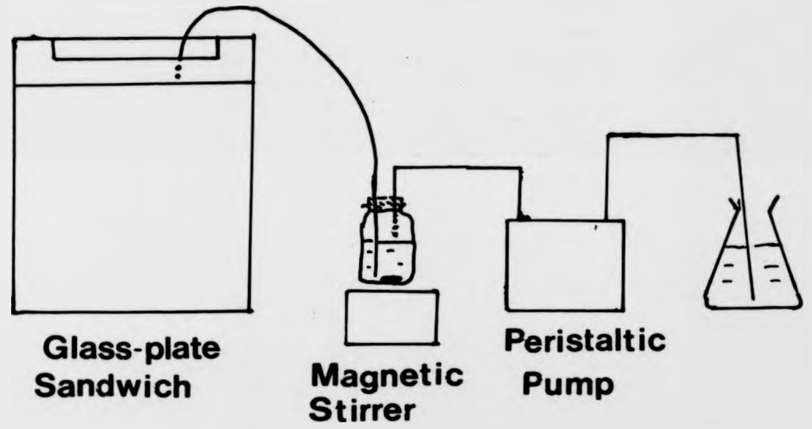
2.13 Gel Electrophoresis of Proteins

2.13.1 10-30% (w/v) polyacrylamide concave gradient gels

This technique employed the ionic detergent, sodium dodecyl sulphate (SDS) to dissociate all proteins into their individual polypeptide subunits. A discontinuous buffer system was used with a stacking gel polymerised on top of the resolving gel (Laemmli, 1970). The gels were cast between glass plates and run in electrophoresis tanks similar to that described by Studier (1973). The pouring of a concave gradient gel is shown diagrammatically in Figure 2.2. For a 10-30% (w/v) polyacrylamide gradient gel of 50 ml total volume, 10 ml of 30% (w/v) acrylamide mixture was placed in the mixing chamber and this was stoppered with a suba seal. The 10% (w/v) acrylamide mixture was pumped into the 30% (w/v) acrylamide mixture using a peristaltic pump and thoroughly mixed using a magnetic stirrer in the mixing chamber. During pouring the volume of liquid in the mixing chamber remained constant and was continuously diluted by 10% (w/v) gel solution. The gradient of acrylamide was pumped between the glass plates which were separated by

Figure 2.2

Diagram of apparatus for pouring concave gradient polyacrylamide gels.



perspex spacers, sealed by polypropylene tubing smeared with vaseline, and held together by bulldog clips. After pouring the gel solution was overlaid with butanol saturated with water and allowed to set. The butanol was then removed and a stacking gel set above the resolving gel with a perspex slot former pushed into the stacking gel. After setting the slot former and polypropylene tubing were removed and the gel placed in the tank for loading.

The gel solutions were prepared as follows:

Resolving gel buffer pH 8.8

Tris (Trizma base) 36.6 g

Concentrated hydrochloric acid 4.13 ml

Distilled water to 100 ml

Stacking gel buffer pH 6.8

Tris (Trizma base) 5.98 g

Concentrated hydrochloric acid 4.13 ml

Distilled water to 100 ml

60% High bis acrylamide stock

Acrylamide 60 g (Eastman Kodak)

Bisacrylamide 1.6 g (Eastman Kodak)

Distilled water to 100 ml

60% low bisacrylamide stock

Acrylamide 60 g

Bisacrylamide 0.3 g

Distilled water to 100 ml

Stacking gel acrylamide stock

Acrylamide 10 g

Bisacrylamide 0.5 g

Distilled water to 100 ml

Reservoir buffer 5 x stock

Tris (Trizma base) 30.2 g

Glycine (B.D.H.) 144 g

Distilled water to 1000 ml

Reservoir buffer

5 x stock 200 ml

10% (w/v) sodium dodecyl sulphate solution (SDS) 10 ml

Distilled water to 1000 ml

30% (w/v) acrylamide mixture - 20 ml

Low bisacrylamide stock 10 ml

75% v/v glycerol 7.3 ml

Resolving gel buffer stock 2.5 ml

10% (w/v) SDS solution 0.2 ml

TEMED (N,N,N,N'-tetramethylenediamene) 4 µl (Eastman Kodak)

This was vortexed, degassed under vacuum in a desiccator, and immediately before pouring 40 µl of 10% (w/v) ammonium persulphate added.

10% (w/v) acrylamide mixture - 50 ml

High bisacrylamide stock	8.3 ml
Distilled water	34.9 ml
Resolving gel buffer	6.25 ml
10% (w/v) SDS	0.5 ml
TEMED	10 µ

This was vortexed, degassed under vacuum in a desiccator and immediately before pouring 100 µl of 10% (w/v) ammonium persulphate solution added.

Stacking gel mixture - 10 ml

Stacking gel acrylamide stock	3.0 ml
Distilled water	4.4 ml
Stacking gel buffer	2.4 ml
10% SDS	0.1 ml
TEMED	5 µl

This was vortexed, degassed under vacuum in a desiccator and immediately before pouring 100 µl of 10% (w/v) ammonium persulphate solution added.

Glycerol was included to produce a density gradient to mimimise mixing due to convection caused by the heat evolved during polymerisation. This avoids the need to include a gradient of polymerisation catalyst (ammonium persulphate) to produce polymerisation first at the top of the gel progressing to the bottom. Electrophoresis was carried out on a perspex gel tank at 4°C in a cold room. Air bubbles were removed from beneath the gel using a syringe fitted with a bent hypodermic needle. Samples containing known amounts of protein or known counts of radiolabel were prepared as follows:

To the sample in a volume of 5-50 µl was added 10 µl 10% (w/v) SDS, 5 µl β-mercaptoethanol, 10 µl 75% (w/v) glycerol, and 5 µl bromophenol blue

10% (w/v) acrylamide mixture - 50 ml

High bisacrylamide stock	8.3 ml
Distilled water	34.9 ml
Resolving gel buffer	6.25 ml
10% (w/v) SDS	0.5 ml
TEMED	10 µ

This was vortexed, degassed under vacuum in a desiccator and immediately before pouring 100 µl of 10% (w/v) ammonium persulphate solution added.

Stacking gel mixture - 10 ml

Stacking gel acrylamide stock	3.0 ml
Distilled water	4.4 ml
Stacking gel buffer	2.4 ml
10% SDS	0.1 ml
TEMED	5 µl

This was vortexed, degassed under vacuum in a desiccator and immediately before pouring 100 µl of 10% (w/v) ammonium persulphate solution added.

Glycerol was included to produce a density gradient to mimimise mixing due to convection caused by the heat evolved during polymerisation. This avoids the need to include a gradient of polymerisation catalyst (ammonium persulphate) to produce polymerisation first at the top of the gel progressing to the bottom. Electrophoresis was carried out on a perspex gel tank at $4^{o}C$ in a cold room. Air bubbles were removed from beneath the gel using a syringe fitted with a bent hypodermic needle. Samples containing known amounts of protein or known counts of radiolabel were prepared as follows:

To the sample in a volume of 5-50 µl was added 10 µl 10% (w/v) SDS, 5 µl β-mercaptoethanol, 10 µl 75% (w/v) glycerol, and 5 µl bromophenol blue

solution (tracker dye). This mixture was boiled in a glass vial for 5 minutes, cooled, then loaded using a Hamilton syringe.

Electrophoresis was overnight for 15 hours at 20 mA and 4°C until the tracker dye reached the bottom of the gel when the gel was removed from the plates fixed and stained as required. The electrophoresis of protein standards of known molecular ratio produced a calibration curve which was linear in the central position (see Figure 2.3) when $\log_{10} M_r$ was plotted against R_f.

2.13.2 Non-denaturing protein gels

The method found to give good resolution of native proteins was adapted from a system described by Sargent and George (1975). The method was adapted to produce a 5 to 15% (w/v) polyacrylamide concave gradient gel in order to try to eliminate the effects of charge on the mobility of native proteins by electrophoresing until proteins were halted by the retarding effect of the increasing acrylamide concentration. Electrophoresis of standards confirmed that separation was on the basis of molecular ratio and gave a calibration curve (see Figure 2.4).

Pouring and running of non-denaturing gels was basically the same as described for the 10-30% polyacrylamide denaturing gels except for the different gel constituents. Plates were cleaned with chromic acid to remove traces of detergent and gel tanks were extensively rinsed in distilled water for the same reason.

Figure 2.3

Calibration curve for 10-30% (w/v) polyacrylamide concave gradient denaturing protein gel.

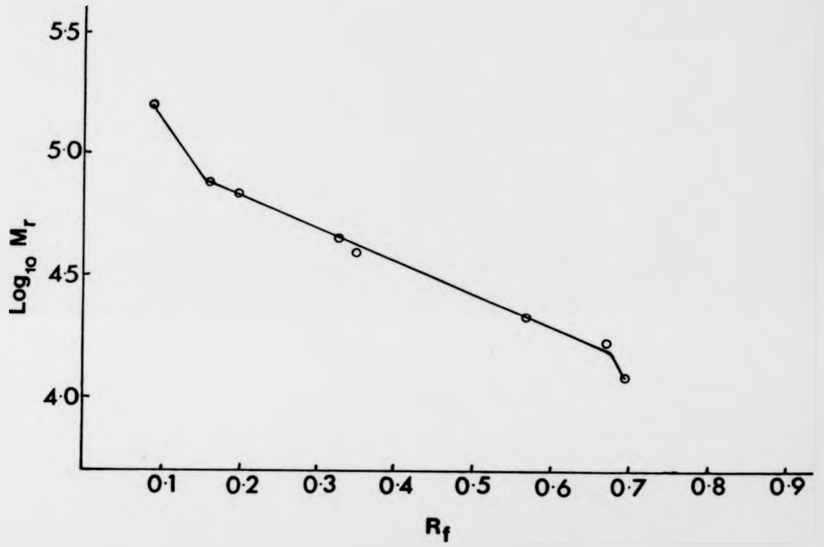
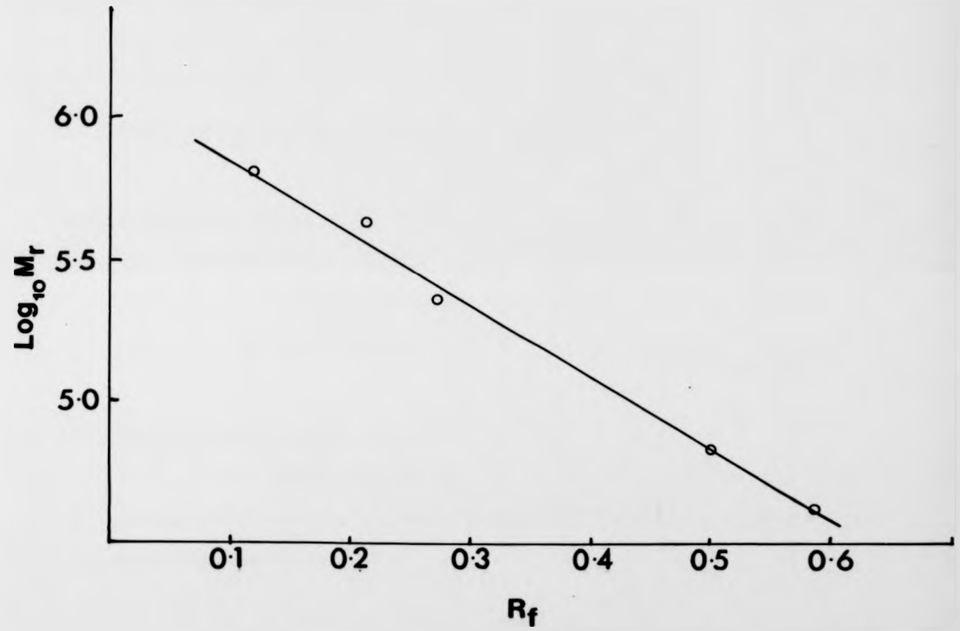


Figure 2.4

Calibration curve for 5-15% (w/v) polyacrylamide concave gradient non-denaturing protein gels.



The gel solutions were prepared as follows:

30% (w/v) acrylamide stock

Acrylamide 28.5 g

Bisacrylamide 1.5 g

Distilled water to 100 ml

Stacking gel acrylamide stock

Acrylamide 10 g

Bisacrylamide 2.5 g

Distilled water to 100 ml

Resolving gel buffer stock pH 8.8

Tris (Trizma base) 36.6 g

Concentrated hydrochloric acid 4.13 ml

Distilled water to 100 ml

Stacking gel buffer stock pH 6.9

Tris (Trizma base) 5.7 g

Concentrated phosphoric acid (H_3PO_4) 1.5 ml (adjust to correct pH)

Distilled water to 100 ml

Reservoir buffer stock 50x

Tris (Trizma base) 30.2 g

Glycine 144 g

Distilled water to 1000 ml

Reservoir buffer

50 x stock 20 ml

Distilled water to 1000 ml

15% (w/v) acrylamide mixture - 20 ml

30% (w/v) acrylamide stock	10 ml
75% v/v glycerol	7.5 ml
Resolving gel buffer	2.5 ml
TEMED	4 µl

This was vortexed, degassed under vacuum in a desiccator, and immediately before pouring 40 µl of 10% (w/v) ammonium persulphate solution added.

4% (w/v) acrylamide mixture - 50 ml

30% (w/v) acrylamide stock	6.7 ml
Distilled water	37.0 ml
Resolving gel buffer	6.25 ml
TEMED	10 µl

This was vortexed, degassed under vacuum in a desiccator, and immediately before pouring 100 µl of 10% (w/v) ammonium persulphate solution added.

Stacking gel acrylamide mixture - 10 ml

Stacking gel acrylamide stock	2.5 ml
Stacking gel buffer stock	1.25 ml
40% w/v sucrose	5.0 ml
Distilled water	1.25 ml
TEMED	4 µl

This was vortexed, degassed under vacuum in a desiccator and immediately before pouring 100 µl of 10% (w/v) ammonium persulphate solution added.

2.13.3 Re-electrophoresis of bands cut from non-denaturing gels on SDS polyacrylamide gels

This technique enabled a band located on a dried gel by autoradiography to be electrophoresed in a denaturing gel to separate individual polypeptides. In practice numerous polypeptides arise from a single band on a non-denaturing gel because several proteins make up each band. However the technique does allow the determination of the native molecular ratio of polypeptides separated on SDS denaturing gels.

The dried strip of gel was first rehydrated in 40% v/v methanol with 7% v/v acetic acid then soaked twice for 15 minutes each in equilibration buffer containing 62 mM Tris-HCl buffer pH 6.8, 2% w/v SDS and 0.5% v/v beta-mercaptoethanol. The rehydrated strip was then sealed into a slot on a 10-30% (w/v) denaturing gel with 1% (w/v) agarose solution in the equilibration buffer. After electrophoresis the gel was stained or autoradiographed to locate the proteins of interest.

2.13.4 Two-dimensional electrophoresis using the O'Farrell method

This technique separates proteins according to isoelectric point in the first dimension and according to molecular ratio by SDS-PAGE in the second dimension. The technique has proved to be capable of resolving 1100 different proteins from Escherichia coli (O'Farrell, 1975) and should be capable of resolving up to 5,000 proteins. Using one-dimensional SDS-PAGE about 100 bands can normally be resolved so each band probably consists on average of about 10 different proteins having similar molecular weights. Protein species representing less than one

part in a million of the total protein can be detected even if they contain as little as one disintegration per minute of ^{35}S radiolabel. The pattern of proteins produced can be visualized either by autoradiography, fluorography or by silver staining and these techniques are discussed later.

The first dimension isoelectric focussing gels were made in 130 mm x 2.5 mm internal diameter glass tubing sealed at the bottom with Parafilm held in place by a short length of plastic tubing. The gels were 100 mm long. The apparatus for isoelectric focussing consisted of a tube gel tank adapted with bored rubber bungs to accommodate the narrow tubes. After isolectric focussing the gels were removed by air pressure from a syringe and plastic tubing and after equilibration loaded onto the stacking gel of a 10-30% (w/v) SDS denaturing gel which provided the second dimension.

Lysates were produced as described previously by sonication then DNAase added to 50 µg ml^{-1}. After 5 minutes on ice solid urea was added to 9 M and 1 volume of lysis buffer A added.

The buffers and solutions were prepared as follows:

A Lysis buffer

9.5 M Urea (ultra pure) 5.7 g per 10 ml

NP-40 (non ionic detergent) 2% (w/v)

Ampholines (LKB) Range 5-7 pH 1.6% (w/v)

Ampholines (LKB) Range 3.5-10 pH 0.4% (w/v)

Beta-mercaptoethanol 5% (v/v)

This buffer was stored as frozen aliquots at -20°C.

B Sonication buffer

0.01 M Tris HCl buffer pH 7.4

5 mM Magnesium chloride ($MgCl_2$)

Pancreatic RNAase 50 $\mu g/ml^{-1}$

C DNAase solution

Pancreatic DNAase 1 mg ml^{-1}

0.01 M Tris HCl buffer pH 7.4

1 mM Magnesium chloride ($MgCl_2$)

This buffer was stored as frozen aliquots at $-20^{o}C$.

D 30% (w/v) acrylamide solution for isoelectric focusing

Acrylamide 28.38 g

Bisacrylamide 1.62 g

Distilled water to 100 ml

E Nonidet P-40 solution

NP-40 10 g

Distilled water to 100 ml

F Ampholines

These were used as supplied by LKB as 40% w/v solutions

G Ammonium persulphate solution

10% w/v solution prepared fresh each day

H Gel overlay solution

8 M urea 4.8 g per 10 ml

This was stored as frozen aliquots at -20°C.

I Anode electrode solution

0.01 M Phosphoric acid solution 1.12 ml concentrated acid/pwr 2L.

J Cathode electrode solution

0.02 M Sodium hydroxide solution 1.6 g per 2 l.

This solution was degassed under vacuum before use.

K Sample overlay solution

9 M urea 5.4 g per 10 ml

Ampholine range 5-7 pH 0.8% (w/v)

Ampholine range 3.5-10 pH 0.2% (w/v)

This solution was stored as frozen aliquots at -20°C.

Buffers and solutions L, M and N were gel buffers and acrylamide solutions for the second dimension gel described in section 2.13.1.

O SDS sample buffer

10% w/v glycerol

5% v/v beta-mercaptoethanol

2.3% w/v Sodium dodecylsulphate (SDS)

0.0625 M Tris-HCl pH 6.8 (0.757 g per 100 ml)

P Agarose gel

1 g of agarose was melted in 100 ml of buffer 'O' then 2 ml of 0.1%

(w/v) Bromophenol blue solution added. This was stored as aliquots at 4°C.

Isoelectric focusing gel mixture - 10 ml

5.5 g urea

1.33 ml Acrylamide stock D

2 ml NP-40 solution E

1.97 ml distilled water

0.4 ml Ampholines range 5-7 pH

0.1 ml Ampholines range 3.5-10 pH

7 µ TEMED

This solution was vortexed, degassed under vacuum then 10 µl of 10% (w/v) ammonium persulphate solution added immediately before pouring. The isoelectric focusing tubes were filled using a syringe fitted with a needle and fine plastic tubing to avoid trapping air bubbles in the tube. The solution was overlayed with gel overlay solution 'H' and allowed to set for 1-2 hours. 'H' was removed and replaced with 20 µl of lysis buffer 'A' and this overlayed with water. After allowing to set for a further 1-2 hours the lysis buffer and water were removed, 20 µl of fresh lysis buffer added, and the tubes filled with 0.02 M sodium hydroxide. The parafilm at the bottom of the tubes was removed and the tubes were placed in the electrophoresis tank. The lower chamber was filled with the Anode electrode solution 'I' and the upper chamber with Cathode electrode solution 'J'. The gels were then pre-run at 200 V for 15 minutes, 300 V for 30 minutes and 400 V for 30 minutes. After the pre-run the power was turned off and the upper reservoir emptied. The lysis buffer and sodium hydroxide solution was removed from the tubes, the samples loaded then overlayed with 10 µl of Sample Overlay solution

'K' and this with 0.02 M sodium hydroxide solution. The chamber was refilled and the gels focused at 400 V for 12 hours and then at 800 V for a further hour. The gels were then extracted into 5 ml of SDS sample buffer 'O' and equilibrated on a shaker for about 2 hours. They were then loaded onto the second dimension gel or frozen in an ethanol dry-ice bath and stored at -70°C.

The second dimension gel was cast using a plate with a bevelled edge to produce a space for the isoelectric focusing gel and the stacking gel was cast to the bottom of this space. The isoelectric focusing gel was placed on a piece of Parafilm and straightened. One ml of agarose solution 'P' was put into the space on top of the second dimension gel and the first dimension gel transferred to this. The second dimension ws electrophoresed as soon as the agarose set. After use, plates and tubes were soaked in chromic acid, rinsed in distilled water, soaked in alcoholic potassium hydroxide solution, rinsed again extensively with distilled water and air dried (O'Farrell, 1975).

2.13.5 Two-dimensional electrophoresis using non-equilibrium pH gradient electrophoresis in the first dimension

The two-dimensional electrophoresis method described in the previous section gave good resolution of proteins with isoelectric points in the range pH 4-7. By using non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension with Ampholines in the range pH 3.5 to 10 most, if not all, of the cellular proteins could be resolved. There was less resolution of acidic proteins than with isoelectric focusing but basic proteins were resolved with NEPHGE. In this method electrophoresis in the first dimension was towards the cathode with the acidic reservoir on top and the basic reservoir at the

'K' and this with 0.02 M sodium hydroxide solution. The chamber was refilled and the gels focused at 400 V for 12 hours and then at 800 V for a further hour. The gels were then extracted into 5 ml of SDS sample buffer 'O' and equilibrated on a shaker for about 2 hours. They were then loaded onto the second dimension gel or frozen in an ethanol dry-ice bath and stored at $-70^{o}C$.

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bottom. Thus in effect the protein sample was loaded at the opposite or acidic end of the gel compared to isoelectric focusing where it was loaded at the basic end. With the NEPHGE system electrophoresis was for a short time such that equilibrium was not reached and thus migration depended both on isoelectric point and molecular ratio with larger proteins not migrating so far irrespective of their isoelectric point.

The majority of the procedures and solutions were as described in the previous section with a few changes in the procedure for the first dimension which are now detailed.

NEPHGE gel mixture - 10 ml

5.5 g urea

1.33 ml Acrylamide stock D

2 ml NP-40 solution E

1.97 distilled water

0.5 ml Ampholines range 3.5 - 10 pH

14 µl TEMED

This solution was vortexed, degassed under vacuum then 20 µl of 10% (w/v) ammonium persulphate added immediately before pouring. After the tubes were filled the solution was overlayed with distilled water and allowed to set for 1 to 2 hours. The samples were then loaded and overlaid with 20 µl of overlay solution which consisted of 8 M urea, 0.8% (w/v) Ampholines range 5-7 pH and 0.2% (w/v) Ampholines range 3.5-10 pH. The tubes were then filled with 0.01 M phosphoric acid. The lower chamber of the electrophoresis chamber was filled with 0.02 M sodium hydroxide solution and the upper chamber with 0.01 M phosphoric acid. The gels were electrophoresed for about 4 hours at 400 V for a total of 1600 V hours. The gels were then treated and the second

dimension run as described in section 2.13.4 (O'Farrell et al., 1977).

2.14 Staining of Polyacrylamide Gels

2.14.1 Coomassie blue staining

The staining solution contained 45% v/v methanol, 10% v/v glacial acetic acid and 0.1% w/v Coomassie Brilliant Blue R250. Gels were immersed in stain for about 5 hours and then destained in 45% v/v methanol, 10% (v/v) glacial acetic acid for as long as was required to produce an acceptable background. This procedure was insensitive needing about 100 µg of total soluble cell protein per track in one dimensional PAGE to produce acceptable results and its main use was to locate non-radioactive standards before autoradiography or fluorography.

2.14.2 Silver staining - method of Switzer

Several silver stain methods have been published since 1979 (Ochs et al., 1981). These have the advantage over the Coomassie blue stain of approximately 100 times greater sensitivity. The original method of Switzer et al. (1979) is complicated, very time consuming, employs the noxious substance glutaraldehyde and care is needed in handling gels during the many solution changes because they become very fragile. It is however very sensitive, about 0.05 µg protein per band, and appears to be relatively free from artifactual bands.

Briefly the method used was as follows with slight adaptations:

Fixation

1) 500 ml methanol, 120 ml acetic acid, 380 ml H_2O - 60 minutes

2) 100 ml ethanol, 50 ml acetic acid, 850 ml H_2O - 20 minutes

3) 1000 ml distilled water - 20 minutes

4) 500 ml 1% (v/v) buffered glutaraldehyde - 15 minutes

5) 1000 ml distilled water - 20 minutes then repeated twice.

Staining

6) 500 ml silver stain solution - 20 minutes

7) 1000 ml distilled water - 15 minutes

8) 1000 ml Switzer reducer solution - 10-30 minutes

Protein bands became visible at this stage therefore staining was continued until a desired level was reached.

Destaining - necessary only if background was yellow.

10) 900 ml Farmers' photographic reducer solution - 5-15 minutes.

11) 1000 ml distilled water - 20 minutes repeated once.

The gels were then photographed using a light box, and soaked in 70% (v/v) methanol, 3% (v/v) glycerol for 10 minutes and dried if required as described in section 2.15.

1% (w/v) buffered glutaraldehyde

480 ml distilled water, 9.5 g sodium tetraborate

20 ml 25% (w/v) glutaraldehyde (E.M. grade) pH 8.0

Silver stain solution

95 ml distilled water, 9.2 ml 1M sodium hydroxide solution, and 9.5 ml 25% (w/v) ammonium hydroxide solution were first mixed. To this was added, slowly, 20 ml of silver nitrate solution containing 3.88 g of silver nitrate. The final clear solution was made up to 500 ml with

distilled water.

Switzer reducer solution

100 ml ethanol, 6 ml of 1% (w/v) freshly made citric acid solution, and 2.5 ml of 3.7% (w/v) formaldehyde solution were made up to 100 ml with distilled water.

Farmers' photographic reducer solution

20 ml of 20% (w/v) sodium thiosulphate solution and 20 ml of 1% (w/v) potassium ferricyanate solution was made up to 900 ml with distilled water.

2.14.3 Silver staining - method of Morrissey

An alternative silver stain procedure (Morrissey, 1981) was much less tedious to perform, used small amounts of silver nitrate and had the advantage that several gels could be stained in one container allowing comparisons in staining pattern from gel to gel. The main disadvantage of the method was the use of 10% (w/v) glutaraldehyde which was both expensive and noxious.

Briefly the method was as follows:

1) 50% (v/v) methanol, 10% (v/v) glacial acetic acid - 30 minutes.
2) 5% (v/v) methanol, 7% (v/v) glacial acetic acid - 30 minutes.
3) 10% (w/v) glutaraldehyde solution - 30 minutes.
4) 1000 ml distilled water - overnight.
5) 1000 ml distilled water - 30 minutes.
6) 200 ml 5 $\mu g\ ml^{-1}$ dithiothreitol solution - 30 minutes.
7) 200 0.1% (w/v) silver nitrate solution - 30 minutes
8) The gel was rinsed with distilled water, then twice with developer,

then soaked in developer until the desired level of staining was reached. The developer contained 100 µl of 37% (w/v) formaldehyde solution in 200 mls of 3% (w/v) sodium carbonate solution.

9) 10 ml of 2.3 M citric acid solution was added directly to the developer to stop the staining process.

10) The gel was washed in several changes of distilled water for 30 minutes.

2.14.4 Silver staining - method of Wray et al.

The third method of silver staining used was that of Wray et al. 1981. This method had advantages of simplicity and rapidity but was found to be less sensitive than the previous methods and occasionally failed entirely. This was probably due to interference by glycerol or glycine which needed to be removed by soaking in several changes of 50% (v/v) methanol before staining. Artifactual bands, presumably deriving from impurities in gel constituents, also appeared to be more noticeable in this method.

Briefly, the method was as follows:

1) 50% (v/v) methanol - 60 minutes.

2) The stain solution was prepared as follows:
0.8 g of silver nitrate was dissolved in 4 ml of distilled water to produce solution A. 21 ml of 0.36% (w/v) sodium hydroxide solution was mixed with 1.4 ml of 14.8 M ammonium hydroxide solution to produce solution B. Solution A was added dropwise to solution B with constant vortexing and finally made up to 100 ml with distilled water.

3) The gel was soaked in the stain solution for 15 minutes.

4) 1000 ml distilled water - 5 minutes.

5) The gel was then soaked in developer solution until bands appeared in not more than 15 minutes. The developer solution was prepared by mixing 2.5 ml of 1% (w/v) citric acid and 0.15 ml of 37% (w/v) formaldehyde solution and making the volume up to 500 ml.

6) The gel was then washed with water and placed in 45% methanol, 10% (v/v) acetic acid to stop stain development.

After staining, by whatever method, gels were photographed using a light box and 35 mm Kodak Panatomic X film ASA 32. A yellow filter improved the contrast with Coomassie Blue stained gels.

2.15 Autoradiography

Prior to autoradiography gels were dried. The wet gel was mounted on 3 MM chromatography paper and dried under vacuum between sheets of silicone rubber on a metal plate heated to 80°C above a water bath. The dried gel was placed in contact with Kodak "No-Screen" X ray film in a film envelope between metal plates and covered with aluminium foil. This was kept in a cold-room and was weighted down if thought necessary. Exposure times varied with the amount of radiolabel loaded but as a rough guide on a one-dimensional gel system 100,000 cpm of ^{35}S L Methionine per track needed about 10 days while on a two-dimensional system 1,000,000 cpm of ^{35}S per gel needed about 10 weeks. (In order to reduce these loadings or times fluorography was undertaken.) Films were developed in Kodak DX-80 developer and fixed in Kodak FX-40 fixer. They were photographed as described previously for gels in section 2.14.

2.16 Fluorography

In this method the gel was impregnated with a scintillant before applying the film. The film was exposed not by beta particles only but also by light generated by the interaction of the beta particles with the scintillant. (This procedure improves the sensitivity for the detection of ^{35}S by ten-fold and allows the detection of ^{3}H on X-ray film.) If the film was pre-flashed to an optical density above background fog of about 0.15 at 540 nm sensitivity was further increased by ten-fold for ^{3}H and four fold for ^{35}S.

The procedure for impregnation with the scintillant PPO (2,5-diphenyloxazole) was as follows:

1) The gel was soaked in 20 times its volume of dimethyl sulphoxide for 30 minutes then repeated with fresh dimethyl sulphoxide.
2) The gel was soaked in 4 times its volume of 22.2% w/v PPO in dimethyl sulphoxide for 3 hours.
3) The gel was soaked in 20 times its volume of distilled water for 1 hour then dried under vacuum as previously described.

The Kodak X-Omat H X-ray film was pre-flashed using a Vivitar 283 flash gun fitted with a power control module. The flash window was covered with an orange filter and a piece of filter paper to diffuse the light. The gel was then applied to the film, taped into position, put into film envelopes between metal plates covered with aluminium foil and exposed at -70°C. (The rationale for pre-flashing and exposure at -70°C is worth noting. Each silver halide grain in the emulsion requires several photons in order to be developable. Each photon of light produces a single silver atom but this is unstable undergoing thermal decomposition back to a silver ion. The production of a second silver atom in the

same halide grain stabilizes the first and the grain becomes developable. At -70°C the single silver atom is more stable than at room temperature so a second photon is more likely to be received before it decays. Preflashing to an appropriate level the film may be hypersensitized by pre-forming one silver atom in each grain but not enough to make most of the grains developable. This increases the sensitivity to radioactivity and avoids reciprocity failure where low levels of radioactivity fail to produce an image because the unstable intermediate decays before a second photon is received. This gives a linear response of the optical density of the image to radioactivity.) Exposure times varied with the amount of radioactivity loaded but typically 24 hours with a loading of 10,000 cpm of ^{35}S on a one-dimensional system or 1 week with a loading of 50,000 cpm of ^{35}S on a two-dimensional system were found to be reasonable. After exposure films were developed as previously described (Bonner and Laskey, 1974; Laskey and Mills, 1975).

2.17 Comparison of Protein Patterns on Two-Dimensional Gels Using a Double-Label Technique

The comparison of protein patterns from two different sources is difficult because of variation between different gels. In the present method two lysates labelled with ^{35}S and ^{3}H respectively were mixed and analysed on a single O'Farrell or NEPHGE gel. Following impregnation with PPO and drying, fluorography detects both isotopes but autoradiography through carbon paper detects only ^{35}S. The use of ^{35}S instead of ^{14}C as described by Choo *et al.* (1980) also allows the ^{3}H proteins to be detected separately when the ^{35}S isotope has decayed to

low levels. Cells were labelled with either ^{3}H L Methionine or ^{35}S L Methionine as previously described and cell extracts prepared. The ratio between ^{3}H counts and ^{35}S counts applied to the gel needed to be adjusted to allow for the lower energy of the ^{3}H particle. In general 3 times as much ^{3}H counts were applied as ^{35}S counts but some gels were run with twice as much and equal counts. Because the ^{35}S labelled proteins must be detected by autoradiography high specific activity material was needed as ideally in the order of 1,000,000 cpm of ^{35}S was needed per gel with about 3 times as much ^{3}H. The limiting factor was the amount of protein that could be loaded and still produce an acceptable two-dimensional gel (in this regard it is worth noting that the O'Farrell method with iso-electric focusing is less susceptible to protein overloading than the NEPHGE system).

Briefly the method involved the preparation of two-dimensional gels and fluorography as previously described in sections 2.13 and 2.16. After the fluorographic exposure the dried gel was covered with black carbon paper which excluded light from the scintillant and the weak ^{3}H particle and exposed again at $4^{o}C$ to produce a ^{35}S autoradiograph using Kodak "No Screen" film. The fluorograph and autoradiograph may be compared visually to observe proteins that are unique to the ^{3}H labelled system. It was possible however to detect protein differences by a photographic technique. Firstly a negative was made of the fluorograph by contact printing onto X-ray film using an enlarger. The autoradiograph was aligned precisely on the fluorograph negative so that the spots covered the complementary "holes" and a second contact print made onto photographic paper. This produced a pattern showing black spots due to proteins unique to the ^{3}H labelled system and white spots on a grey background due to proteins that were found in the ^{35}S labelled

system or were common to both (see Figure 2.5). A similar method of comparing double label autoradiographs using ^{35}S Methionine and ^{35}Se Selenomethionine has recently been published (Lecocq et al., 1982).

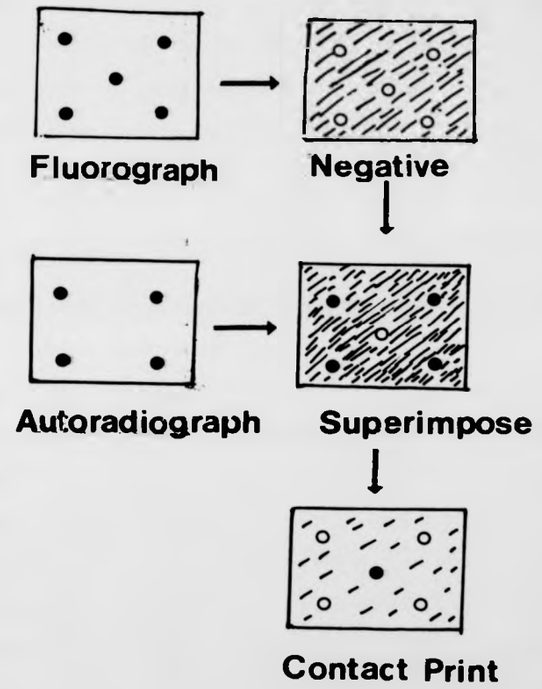
It was also possible to obtain a fluorograph showing the ^{3}H labelled proteins only after the passage of a year when the ^{35}S had largely decayed. This fluorograph was compared to the original fluorograph showing both ^{35}S and ^{3}H labelled proteins and to the autoradiograph showing ^{35}S labelled proteins only. In the latter case negatives were prepared from both fluorograph and autoradiograph to allow photographic comparison with the other. In this way proteins unique to either ^{35}S or ^{3}H labelled systems could be detected.

2.18 Protein Purification by Electrophoresis

The purification of protein to be used in antibody production was undertaken by preparative electrophoresis. Cell lysates were electrophoresed on a 10-30% (w/v) polyacrylamide SDS gel system together with some cell lysate labelled with ^{35}S L Methionine to a high specific activity. After electrophoresis the gel was covered with cling film to protect the X-ray film and an autoradiograph made of the gel which remained on a glass plate. The film was taped into position and its location marked either with pin holes or radioactive ink. After exposure and development of the X-ray film the band of interest was cut from the film to produce a template which could be placed over the gel enabling the band to be cut out with a scalpel. Protein was removed from the gel strip by upward electrophoresis through a stacking gel (Mendel-Hartvig, 1982). This method used the same buffers and equipment described for 10-30% SDS gels. A supporting gel in running buffer was

Figure 2.5

Photographic comparison of double labelled two-dimensional gels.



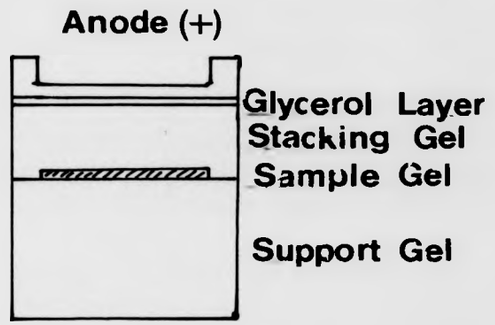
first cast, the gel strip to be eluted was placed on this after first being incubated briefly with 0.5% (w/v) bromophenol blue, and then a stacking gel of about 2 cm cast on top of the gel strip (see Figure 2.6). The electrophoresis was conducted with the anode pole on top at 20 mA at 4°C. The protein was electrophoresed through the stacking gel and collected in an overlay of 2 ml of 30% (v/v) glycerol in stacking gel buffer visualised by the bromophenol blue. The sample was subsequently removed with a syringe.

2.19 Preparation of Antisera

1 ml of protein solution was emulsified with an equal volume of complete Freund's adjuvant by pumping in and out of a 2 ml glass hypodermic syringe until a stiff, white emulsion was produced. The emulsion was injected into a white New Zealand rabbit subcutaneously. The procedure was repeated after 1 month using incomplete adjuvant and again after 2 months. After 3 months the rabbit was bled. The rabbit was wrapped in a cloth exposing one ear and the top of the head and the ear warmed with a reading lamp 30 cm above it. The nick was made in a marginal ear vein and blood collected in a small bottle. About 20 ml of blood may be obtained at each bleeding. The blood clot was prevented from adhering to the walls of the bottle by being moved with a Pasteur pipette and kept overnight in a refrigerator. The serum was then pipetted from the clot and centrifuged at 1500 g for 20 minutes to remove red blood cells. The serum was then stored in 1 ml aliquots at -20°C.

Figure 2.6 (from Mendel-Hartvig 1982)

Elution of protein from polyacrylamide gel by upward electrophoresis through a stacking gel.



2.20 Immunoprecipitation of Proteins

Immunoprecipitation for radioimmune assay of protein was performed by a method described by Lagenaur and Agabian (1978) and Smit and Agabian (1982). Samples were adjusted to 10 mM EDTA, 0.5% (w/v) Triton X-100, 10 mM Tris-HCl pH 7.5, 0.15 M NaCl after addition of 50 µl of unlabelled cell lysate containing approximately 5 mg ml^{-1} total protein to act as carrier. An equal volume of antisera was added to each sample and the precipitation reaction incubated at 37°C for 20 minutes then 4°C for 12 hours. The mixture was then layered onto 0.5 ml of a solution containing 0.15 M NaCl, 0.15 M sodium phosphate pH 7.2, 10 mM EDTA, 0.5 (w/v) Triton X-100 and 1 M sucrose. This was centrifuged at 12000 g for 10 minutes in a microcentrifuge. The supernatant was removed and the pellet washed once in the same solution then solubilized for SDS polyacrylamide gel electrophoresis as previously described in section 2.13.1. The radiolabelled samples were detected by autoradiography or fluorography as previously described in sections 2.15 and 2.16.

2.21 Preparation of ribosomes

Ribosomes were prepared by the method of Muto *et al*. (1974) with minor alterations. Cells were broken by two passages through a French pressure cell at a pressure of 83 x 10^6 Pa suspended in 0.01 M Tris-acetate pH 7.8, 0.01 M magnesium acetate, 0.06 M ammonium acetate, 0.006 M beta-mercaptoethanol. Cell debris was removed by low-speed centrifugation for 10 minutes at 18,000 g. The lysate was treated with 10 µg ml^{-1} DNAase and centrifuged for 30 minutes at 30,000 g. Ribosomes were sedimented from the supernatant by centrifugation at 240,000 g in an MSE 6 x 14 ml swingout rotor for 2 hours. The pellet was resuspended in

0.02 M Tris-acetate pH 7.8, 0.02 M magnesium acetate, 0.05 M ammonium chloride, 0.006 M beta-mercaptoethanol, clarified by low speed centrifugation and sedimented through an equal volume of 30% w/v sucrose in the same buffer at 240,000 g for 12 hours in an 6 x 14 ml swingout rotor.

2.22 Preparation of Membranes

Membranes were prepared by the method of Ito et al. (1977) slightly modified. Cells were washed with 0.03 M Tris-HCl pH 8.1 and suspended in 20% (w/v) sucrose in the same buffer. The suspension was incubated in an ice bath for 30 minutes with 1/10 volume of 1 mg ml^{-1} lysozyme freshly dissolved in 0.1 M EDTA pH 7.3. The preparation was then sonically disrupted by ten 15 second bursts on a sonicator at 1 minute intervals while being cooled in an ice-methanol mixture. The lysate was diluted with an equal volume of 3 mM EDTA pH 7.3 and centrifuged at 2,000 g for 10 minutes to remove unbroken cells and debris. The supernatant was layered onto 7.5 ml of 15% (w/v) sucrose, 3 mM EDTA with a cushion of 70% (w/v) sucrose, 3 mM EDTA at the bottom and centrifuged at 250,000 g in an MSE 6 x 14 ml swingout rotor for 1 hour. Membranes were collected from the 15-70% (w/v) sucrose interphase region.

2.23 Determination of DNA of Bacterial Cells

The colorimetric diphenylamine method was used. 20 to 60 mg of bacterial cells were suspended in 5 ml of ice-cold 0.25 M perchloric acid and incubated for 30 minutes on ice with shaking followed by centrifugation at 5,000 g for 10 minutes. The cells were suspended in 4

ml of 0.5 M perchloric acid and heated on a water bath at 70°C for 15 minutes then centrifuged. This procedure was repeated twice with 3 ml volumes, and the extracts made up to 10 ml. A 2 ml aliquot of this extract was mixed with 2 ml of diphenylamine reagent using a reagent blank containing 0.5 M perchloric acid. The samples were incubated overnight at 30°C and the optical density measured at 600 nm using 1 cm cells. DNA standards were prepared by dissolving calf-thymus DNA in 5 mM sodium hydroxide solution to give 400 μg ml^{-1}. Working standards were prepared by mixing measured volumes of the stock solution with an equal volume of 1 M perchloric acid and heating for 15 minutes to 70°C. The diphenylamine reagent was prepared by dissolving 1.5 gms of steam distilled diphenylamine in 100 ml of redistilled glacial acetic acid and adding 1.5 ml of concentrated sulphuric acid. On the day of use 0.1 ml of aqueous acetaldehyde (16 mg ml^{-1} was added for each 20 ml of reagent (Herbert et al., 1971)).

2.24 Determination of RNA of Bacterial Cells

The colorimetric orcinol method was used. 20-60 mg of bacterial cells were extracted with 95% (v/v) ethanol at 0°C followed by 70% (v/v) ethanol plus 0.1% (w/v) perchloric acid at 0°C and boiling ethanol-ether (3:1) to remove lipids. Two brief extractions with 0.2 M perchloric acid at 0°C removed acid-soluble material. The residue was extracted with 1 M perchloric acid for 18 hours at 4°C and washed twice with 1 M perchloric acid. The combined extracts contained the RNA, and were made up to 10 ml. 1 ml of the extract was added to 3 ml of orcinol reagent heated in a boiling water bath for 20 minutes, cooled and made up to 15 ml with n-butanol and the optical density at 672 nm measured in 1 cm

cells against a reagent blank. Standards were prepared using yeast RNA. The orcinol reagent was prepared immediately before use and contained 2.0 mM ferric chloride, 7.2 M hydrochloric acid and 0.15% (v/v) orcinol (Herbert et al., 1971).

2.25 Determination of ATP Levels in Bacterial Cells

Nucleotides were extracted from cells at 100°C in a water bath in 5 ml of Tris-HCl buffer (0.02 M, pH 7.75) for 5 minutes then cooled on ice (Emala and Weiner, 1983). The extracts were made up to 20 ml and 100 µl aliquots assayed by the luciferase enzyme system (Kimmich et al., 1975). Assays were performed in scintillation vials containing 0.9 ml of assay medium, 100 µl of sample and 10 µl of activated extract. The count cycle of a Packard Tri-Carb scintillation counter was started 20 seconds after addition of the extract and the sample counted for 30 seconds at a Gain setting of 25%. Standards containing from 10 to 100 pmoles of ATP were assayed in the same manner and the counts per minute recorded plotted against the square of ATP concentration which produced a straight line graph. Background counts without sample and without luciferase were also performed. The assay medium contained 5 mM sodium arsenate, 4 mM magnesium sulphate and 20 mM glycyl-glycine, pH 8.0. Luciferase Luciferin was obtained from Sigma (Product No. L-0633) and reconstituted as instructed.

3 RESULTS AND DISCUSSION

3.1 Growth Characteristics of Rhodomicrobium vannielii

The growth of a culture of R. vannielii was followed by recording optical density, cell carbon and the pH of the medium. 5 litres of pyruvate-malate medium was inoculated with 20 ml of a late log-phase culture. The total carbon, cell carbon, inorganic carbon and residual organic carbon were followed using the carbon analyser as described in section 2.9. The results are shown in Figure 3.1. The increase in cell carbon closely parallels the increase in optical density and stationary phase is reached after about 120 hours. The residual organic carbon in the medium, originally pyruvate and malate falls during logarithmic growth but does not fall below 100 ppm indicating that some catabolites are produced that are not assimilable into cell carbon. The inorganic carbon in the medium, representing carbonates, increased to a level of 250 ppm by stationary phase, representing a waste product also not assimilated into cell carbon. About 60% of the total carbon added as pyruvate and malate was converted to cell carbon. The pH of the medium increased steadily during growth as the acidic pyruvate and malate anions were converted to cell carbon. The phosphate buffer added apparently provides little buffering to this pH change and its major function is to provide phosphate for growth. As the culture is a closed system the total carbon should remain constant but a slight decline during growth followed by an increase in the stationary phase was seen consistently. This is probably due to the release of carbon-dioxide into the gas-phase during growth and its uptake during the stationary

Figure 3.1 Growth characteristics of a batch culture

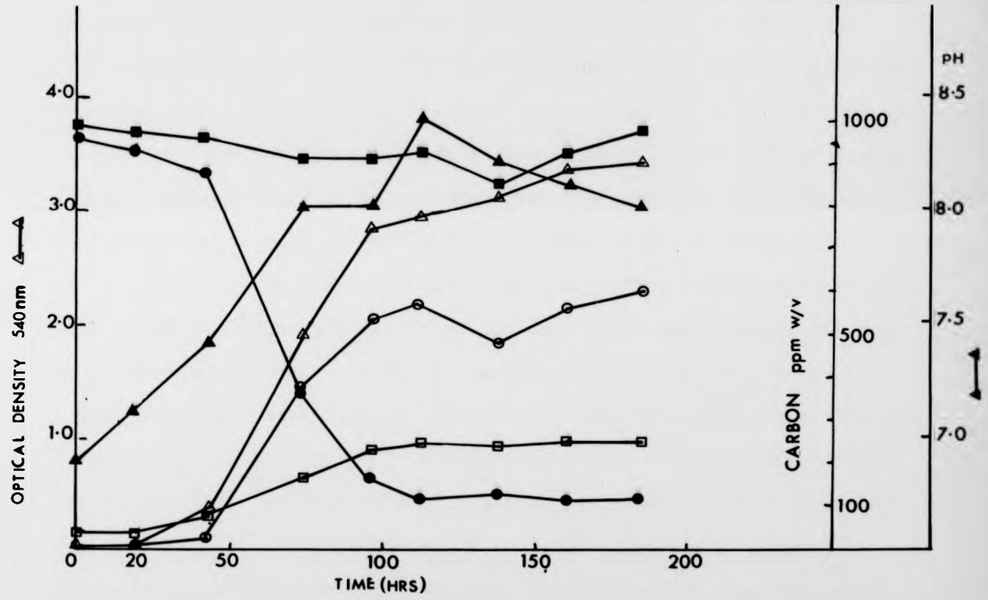
■—■ Total carbon

●—● Organic carbon

○—○ Cell carbon

⊡—□ Inorganic carbon (carbon dioxide)

Carbon levels were measured during the growth of a batch culture of Rhodomicrobium vannielii using a carbon analyser. The optical density and pH of the medium were also recorded.



phase correlating with the observed changes in pH.

3.2 Protein, RNA and DNA Synthesis During Swarmer Cell Differentiation

The protein, RNA and DNA content of homogeneous swarmer cells was measured during development by the colorimetric methods described in sections 2.12, 2.23 and 2.24. Cell numbers were estimated using the Coulter Counter and amounts per cell calculated, the results being shown in Figure 3.2.

Protein and RNA synthesis began after a short lag but DNA synthesis did not commence until stalk synthesis had started after about 3½ hours. This period represents the "I" phase mentioned in the Introduction at section 1.2(b)(x) as the time needed for the maturation of a swarmer cell before DNA synthesis can be initiated. The slower rate of increase in protein during stalk synthesis correlates with a similar change of slope in optical density (A_{540}) (Whittenbury and Dow, 1977). As will be shown in section 3.6 the rate of incorporation of ^{35}S L methionine into protein actually increases during stalk synthesis so the slowing in the rate of increase in protein measured colorimetrically may reflect increased protein turnover at this time. However pulse labelling with ^{35}S L methionine followed by a chase with 100 µM L methionine failed to show loss of radioactivity from protein.

The measurement of nucleic acid synthesis by the incorporation of ^{3}H adenosine into trichloroacetic acid precipitable material during the differentiation was reported by Potts and Dow (1979). DNA synthesis was found only after stalk synthesis had started but RNA synthesis began immediately in agreement with the results obtained colorimetrically

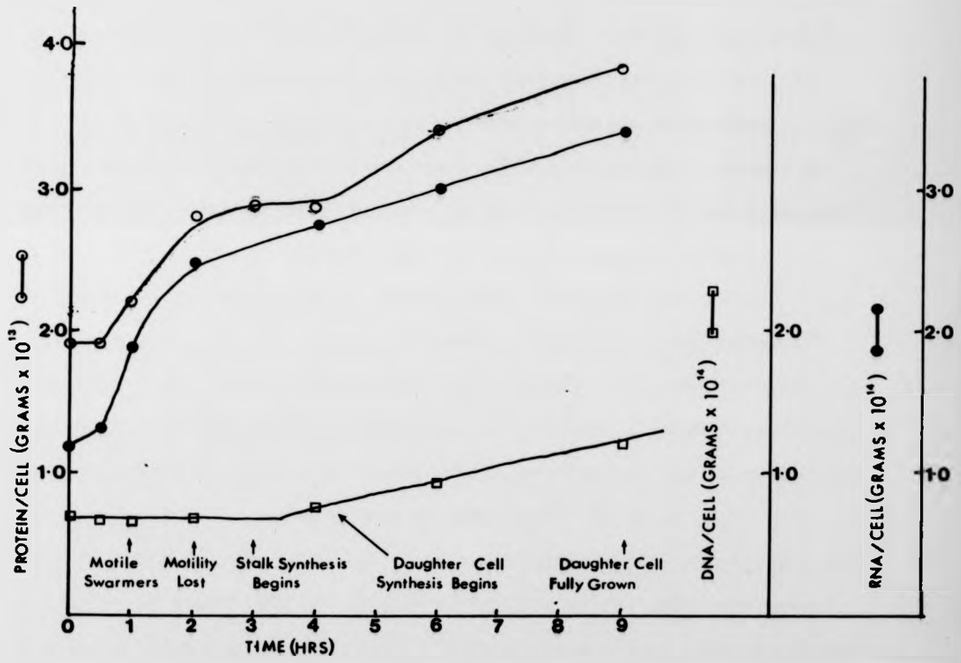
Figure 3.2 Protein, RNA and DNA synthesis during swarmer cell differentiation

Protein/cell

RNA/cell

DNA/cell

Protein, RNA, and DNA were measured colorimetrically during the differentiation of synchronised swarmer cells and expressed in terms of grams per cell using cell numbers derived from the Coulter Counter. Morphological stages were observed by microscopy.



presented here. The advantage of the colorimetric method is that macromolecular synthesis can be seen in relation to the total amount per cell and in this regard Figure 3.2 shows that when the daughter cell is fully grown protein and DNA per cell has doubled. However RNA per cell has almost tripled so it appears that the differentiated stalk cell has more ribosomes than a swarmer cell. Ribosomal RNA is not synthesised in the swarmer cell when differentiation is inhibited by low light intensity (C. Oakley, unpublished data). Some protein synthesis occurs in the swarmer cell when inhibited but presumably sufficient ribosomes are present at division to sustain this low level (see section 3.6).

In Escherichia coli the level of rRNA synthesis is coupled to cell growth at medium and fast growth rates when most of the ribosomes are involved in protein synthesis with few free ribosomes (Jinks-Robertson et al., 1983). These authors suggest that free ribosomes produce feedback inhibition on the transcription of rRNA but others have suggested that the level of ppGpp synthesis controls rRNA synthesis (Richter et al., 1979; Lagosky and Chang, 1980). Ribosomal protein synthesis involves feedback inhibition by specific ribosomal proteins so the production of ribosomes can be precisely regulated (Jinks-Robertson and Nomura, 1982). Seven copies of rRNA genes are found in E. coli (Zubay, 1980) but the addition of more plasmid-encoded rRNA operons does not increase total rRNA transcription and the extra copies are needed simply to allow the maximum growth rates to be achieved (Jinks-Robertson et al., 1983). The budding bacterium Caulobacter crescentus contains only two copies of the *r*DNA sequences consistent with its slower growth rate (Ohta and Newton, 1981). At very low growth rates the coupling between growth rate and ribosome content breaks down and a high proportion of free ribosomes appears in the cell (Norris and Koch,

1972). This is not explicable on the basis of feedback regulation so other controls may be involved.

In R. vannielii sufficient ribosomes are present in the inhibited swarmer cell to sustain protein turnover but new synthesis does not occur. When the block to differentiation is removed (i.e. by light) rRNA increases rapidly in parallel with protein synthesis suggesting some form of control by feedback.

3.3 Changes in Coulter Counter Profile During the Development of an Homogeneous Population of Swarmer Cells

An homogeneous population of swarmer cells was selected from a 3 day old culture by glass-wool filtration as described in section 2.5. Photomicrographs of the heterogeneous culture before synchronisation showing multicellular arrays and swarmer cells and also of the homogeneous swarmer cells after synchronisation are shown in Figures 3.3 and 3.4. The swarmer cells were incubated under the same conditions as the original culture and Coulter Counter profiles and cell counts taken at intervals. Figure 3.5 shows the change in profile size as the motile swarmer cells differentiate to become pairs of cells after 5 hours. After 20 hours differentiation motile swarmer cells reappeared in the culture producing a peak at the original position (Figure 3.6). If the inverse of amplification is altered from 1/2 to 4 another peak can be obtained representing arrays of 8 to 10 cells produced from the original swarmer cells (Figure 3.7).

Cell numbers are shown in Table 3.1 and indicate that the number of arrays produced after 20 hours is approximately equal to the original

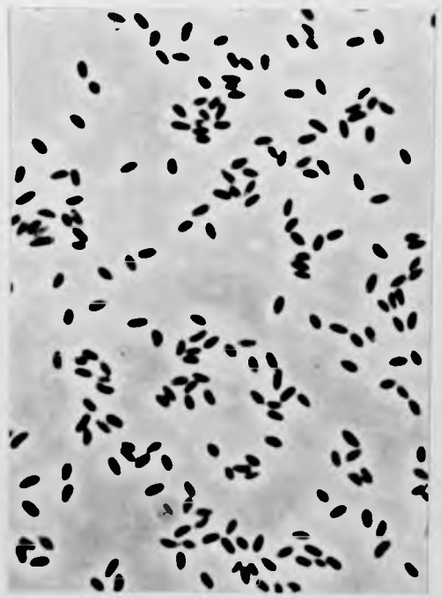
Figure 3.3

Phase contrast micrograph of a 3 day old heterogeneous culture showing multicellular arrays and swarmer cells. Swarmer cells are arrowed.

Figure 3.4

Synchronised swarmer cells from the same culture.

Synchronisation of swarmer cells by selective filtration produces an homogeneous population.



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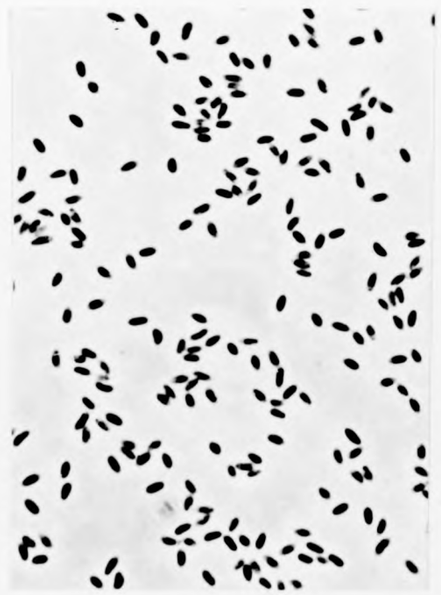


Figure 3.5

Coulter Counter size distribution profiles of differentiating swarmer cells.

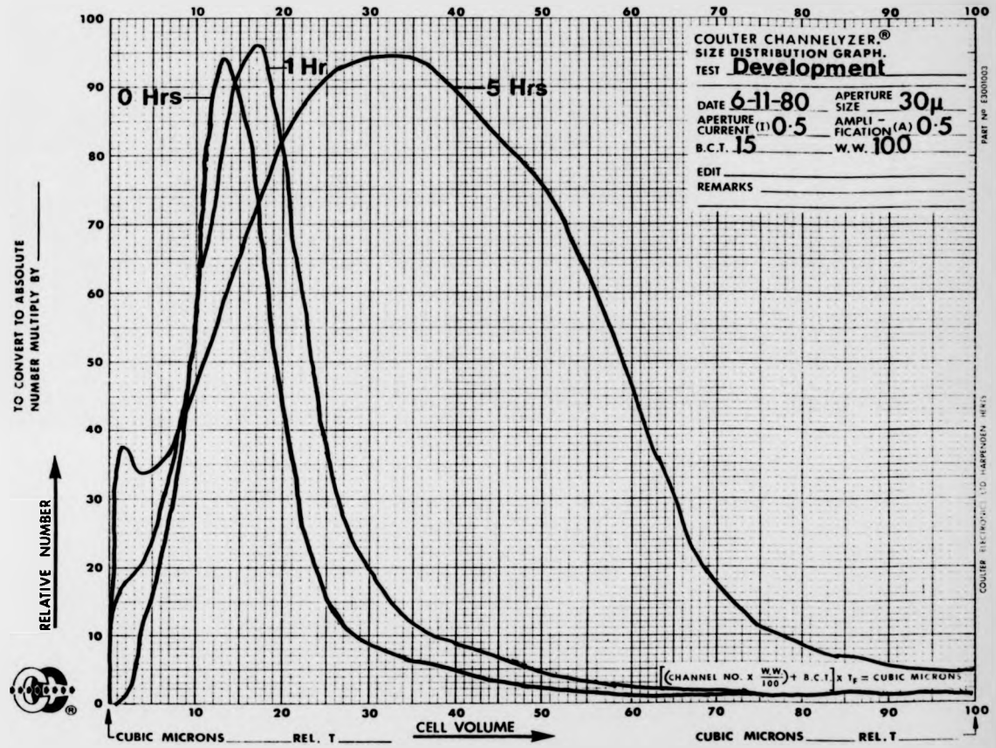


Figure 3.6

Coulter Counter size distribution profile showing a peak of newly released swarmer cells after 20 hours incubation.

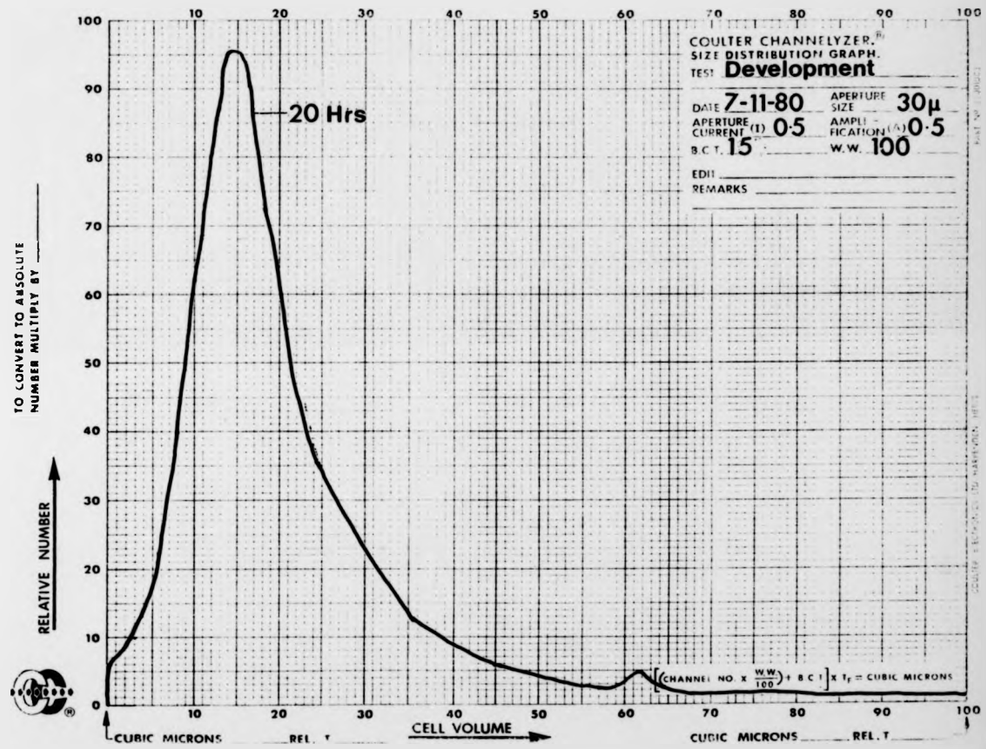


Figure 3.7

Coulter Counter size distribution profile showing a peak of multicellular arrays (note lower amplification) after 20 hours incubation.

Table 3.1

Time - hours	Cells ml^{-1}	Optical Density 540 nm
0	3.46×10^7	0.190
1	3.47×10^7	0.206
2	3.36×10^7	0.215
5	3.05×10^7	0.247
20	2.78×10^7 (arrays)	0.488
	6.01×10^7 (swarmer cells)	

number of swarmer cells. This provides evidence for the synchronous differentiation of the homogeneous population under the increased light intensity provided when the multicellular arrays are removed by filtration.

As shown in section 3.1 about 40% of the original organic carbon remains 3 days after inoculation and about two-thirds of this is used before the culture reaches stationary phase, i.e. the filtered culture is not nutrient limited although there may be other limitations. The synchronous growth of the swarmer cells when the multicellular arrays are removed suggests that light has been the limiting factor in inhibiting their differentiation in the heterogeneous culture. The data in section 3.1 also indicates that it is important to select swarmer cells for synchronous culture studies from the mid-exponential phase at an O.D. of about 2.0. After 100 hours growth of a batch culture 90% of the original organic carbon has been utilized - the remaining 10% is not used - consequently swarmer cells selected at this time do not differentiate as far as multicellular arrays but usually only produce a single daughter cell.

3.4 Changes in the Proportion of Swarmer Cells during the Growth of a Heterogeneous (Complex) Culture

A 5 litre culture vessel was inoculated with 1% v/v of a stationary phase culture. Swarmer cells were counted at intervals using the Coulter Counter with settings of; aperture current 1/2, 1/amplification 1/2, base channel threshold 15, and window width 40, which includes the entire peak of swarmer cells but excludes pairs of cells and

multicellular arrays. The total cell number was estimated from the protein content of the culture using a value for the protein content of an individual cell. This was estimated from the protein content of homogeneous swarmer cells counted using a Coulter Counter. From these figures the number of stalked cells and swarmer cells were calculated for each sample. Starting wtih an inoculum of 5.24×10^6 stalked cells ml^{-1} and 8.97×10^5 swarmer cells ml^{-1}, the number of swarmer cells drops to 6×10^5 ml^{-1} after 150 minutes then rises to 2.1×10^8 ml^{-1} in the stationary phase. The number of stalked cells increases exponentially from inoculation to reach 1.2×10^9 ml^{-1} in the stationary phase. The proportion of swarmer cells falls from the inoculum value of 0.146 to reach a minimum of 0.009 then rises rapidly in the late exponential phase to 0.17 (Figure 3.8).

Dow and France (1980) found that the doubling time of a heterogeneous exponential phase culture was dependent on the light intensity with defined nutrient concentrations. By plotting the reciprocal of doubling time against the reciprocal of light intensity, extrapolation to infinite light intensity gave a theoretical minimum doubling time of 4.9 hours. In the dark doubling time approached infinity as the cells were unable to grow anaerobically without light.

Dow and France (1980) also found the initial decrease in swarmer cell numbers on inoculation into fresh medium and implicate increased light intensity and low carbon-dioxide concentration in producing a cessation of swarmer cell formation. The decrease in the proportion of swarmer cells in the culture can also be explained on the basis of a release from inhibition of differentiation under increased light intensity. This interpretation is supported by the synchronous development of homogeneous swarmers selected by glass-wool filtration

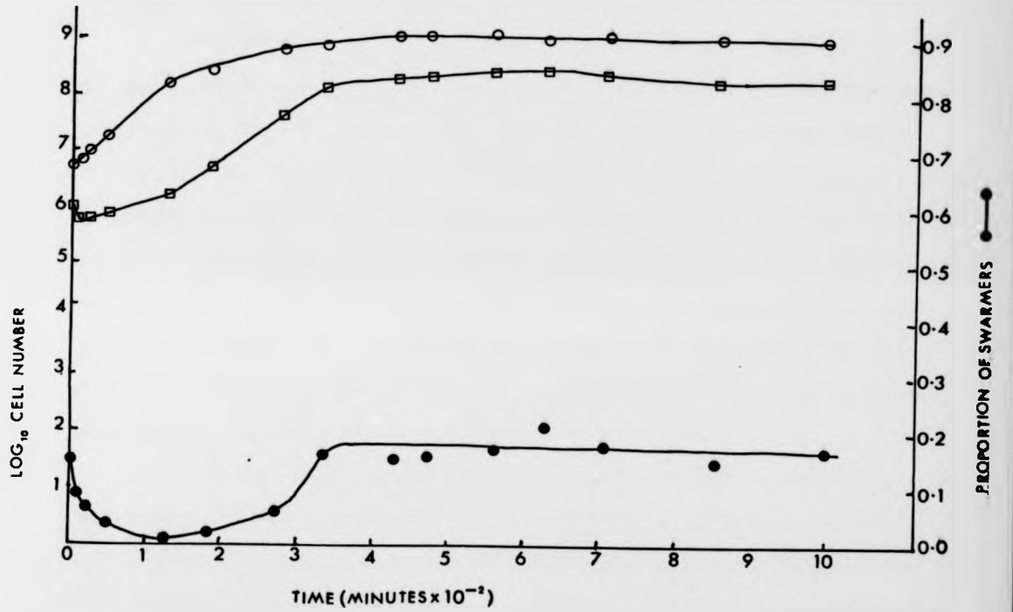
Figure 3.8 Differential cell numbers during growth of a heterogeneous batch culture

○—○ Number of stalked cells ml^{-1}

□—□ Number of swarmer cells ml^{-1}

●—● Proportion of swarmer cells.

Differential cell numbers were estimated by Coulter Counter analysis and protein content.



discussed in section 3.3.

In the late exponential phase the proportion of swarmer cells increases again and this can be explained if the swarmer cells are inhibited from differentiating into stalked cells by a reduction in light intensity caused by the increase in optical density of the culture - dependent on the total number of cells present.

A model based on this hypothesis shows that the growth characteristics can be modelled when various parameters are inserted and cell numbers and cell proportions calculated and plotted using a BBC Microcomputer. The mathematical basis of the model and computer programming is presented in Appendix 4. Figure 4.5 shows the computer model of the differential cell numbers during growth of a heterogeneous culture with parameters chosen to give the best fit to the experimental results in Figure 3.8. Inhibition of swarmer cell differentiation in this model begins to take effect at 1×10^8 cells ml^{-1} and this produces the increase in cells in the late exponential phase.

The inhibition of swarmer cell differentiation can be regarded as an increase in the variable "I" phase, referred to in section 1.2(b)(x), and consequently the increase in the proportion of swarmer cells as the cell number, cell carbon and optical density increase (see section 3.1) implies that the "I" phase is directly related to the optical density of the culture. When the multicellular arrays were removed by selective filtration (see section 2.5) the homogeneous swarmer cells differentiated (see section 3.3), but as is shown later (section 3.6) they do not differentiate if maintained under a dark regime.

The conclusion is that light intensity controls the initiation of swarmer cell differentiation, or in other words the length of an "I" phase, and that this causes the increase in the proportion of swarmer

cells in late log phase.

The computer model of growth shown in Figure 4.5 includes a parameter which increases the ratio of swarmer cells to stalked cells produced in late log phase but its value of 2×10^{10} cells ml^{-1} in this example means that its effect is marginal and the increase in swarmer cell numbers is produced by inhibition of development.

Dow and France (1980) suggest that increased carbon-dioxide tension in late log phase increases the rate of formation of swarmer cells and found that when pH was controlled by the addition of gaseous carbon-dioxide a simplified cell cycle was produced giving constitutive swarmer cell formation.

If in the model the parameter which alters the ratio of swarmer cells to stalked cells produced is reduced to 1×10^{9} cells ml^{-1}, i.e. the same order of magnitude as cell numbers in the late log phase culture the number of swarmer cells increases to that seen in a simplified culture (Figure 4.11).

Thus there is some evidence that increasing carbon-dioxide tension in late log phase stimulates production of swarmer cells while reduced light intensity inhibits their development.

3.5 Incorporation of ^{35}S L Methionine into a Heterogeneous Culture of Rhodomicrobium vannielii

A 50 ml 1 day old subculture was added to 50 ml of fresh medium plus buffer and gassed with nitrogen for 10 minutes. ^{35}S methionine (>300 Ci $mmol^{-1}$) was added to give either 1 or 2 $\mu Ci\ ml^{-1}$. At intervals 1 ml samples were taken and L-Methionine added to 1 mM to prevent

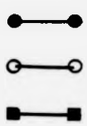
further incorporation of ^{35}S L Methionine. The samples were incubated at 30°C for 5 minutes then 3 x 100 µl aliquots added to 2 ml of ice-cold 5% (w/v) trichloracetic acid and radiolabel determined as described in section 2.10. Figure 3.9 shows that the initial rate of incorporation is rapid and independent of the concentration of radiolabel. The addition of unlabelled L Methionine to 10 µM slowed the initial rate of incorporation by 75% and also reduced the eventual incorporation by 50%. The incorporation of ^{35}S L methionine into Rhodomicrobium vannielii cells is rapid and depressed by the presence of unlabelled L methionine. Pre-exposure of the cells to L methionine in order to activate transport mechanisms would not appear to be necessary.

3.6 Incorporation of ^{35}S L Methionine into an Homogeneous Population of Swarmer Cells and the use of this to Follow Protein Synthesis

^{35}S L methionine was added to 100 ml aliquots of synchronised swarmer cells at the rate of 0.5 µCi ml^{-1}. One sample was maintained at 30°C in the light and another kept in the dark under otherwise identical incubation conditions. At intervals 1 ml samples were taken and L methionine added to 1 mM to prevent further incorporation. The samples were incubated at 30°C for 5 minutes then 3 x 250 µl aliquots added to 2 ml of ice-cold 5% (w/v) trichloracetic acid and radiolabel determined as described in section 2.10.

Figures 3.10 and 3.11 are Coulter-Counter cell size distributions which show that the homogenous population of swarmers did not differentiate even after 2 days under dark conditions while under light conditions the cells differentiated to produce pairs and multicellular

Figure 3.9 Incorporation of ^{35}S L-methionine into a heterogeneous culture



2 µCi ml_{-1} ^{35}S L-methionine (>300 Ci $mmol^{-1}$)

1 µCi ml^{-1} ^{35}S L-methionine

1 µCi ml^{-1} ^{35}S L-methionine + 10 µM L-methionine

Incorporation of ^{35}S L-methionine was determined by adding 100 µl aliquots to 2 ml ice-cold 5% trichloroacetic acid, collectingthe insoluble material on GFC papers and scintillation counting as described in section 2.10.

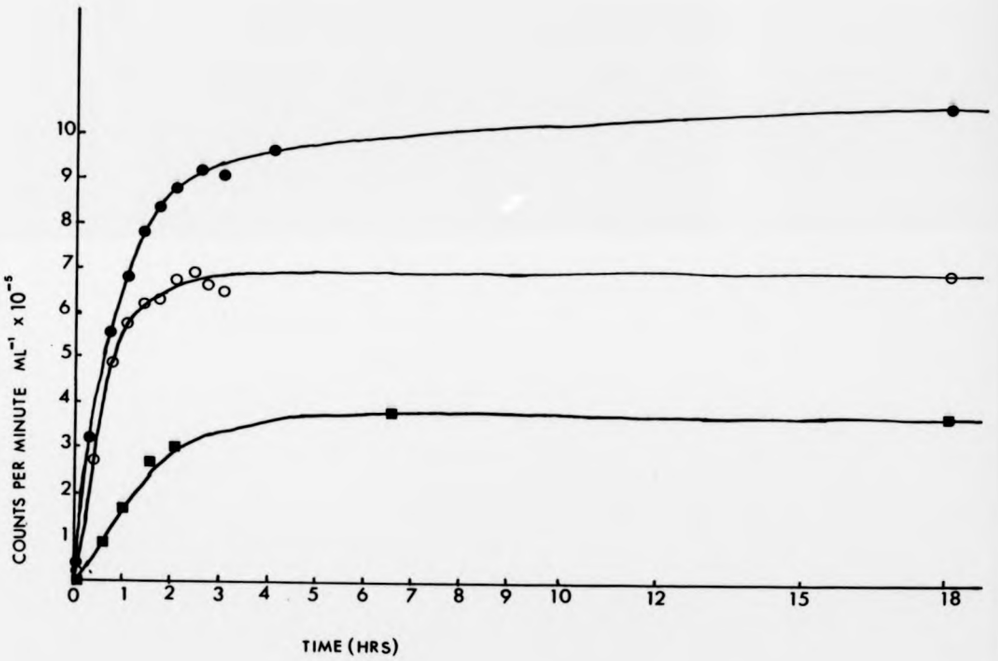

COUNTS PER MINUTE ML^{-1} x 10^{-5}
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
12
15
18
TIME (HRS)

Figure 3.10

Coulter Counter size distribution profile showing synchronised swarmer cells after 2 days incubation in the dark. This indicates that swarmer cells remain inhibited and do not differentiate under light limitation.

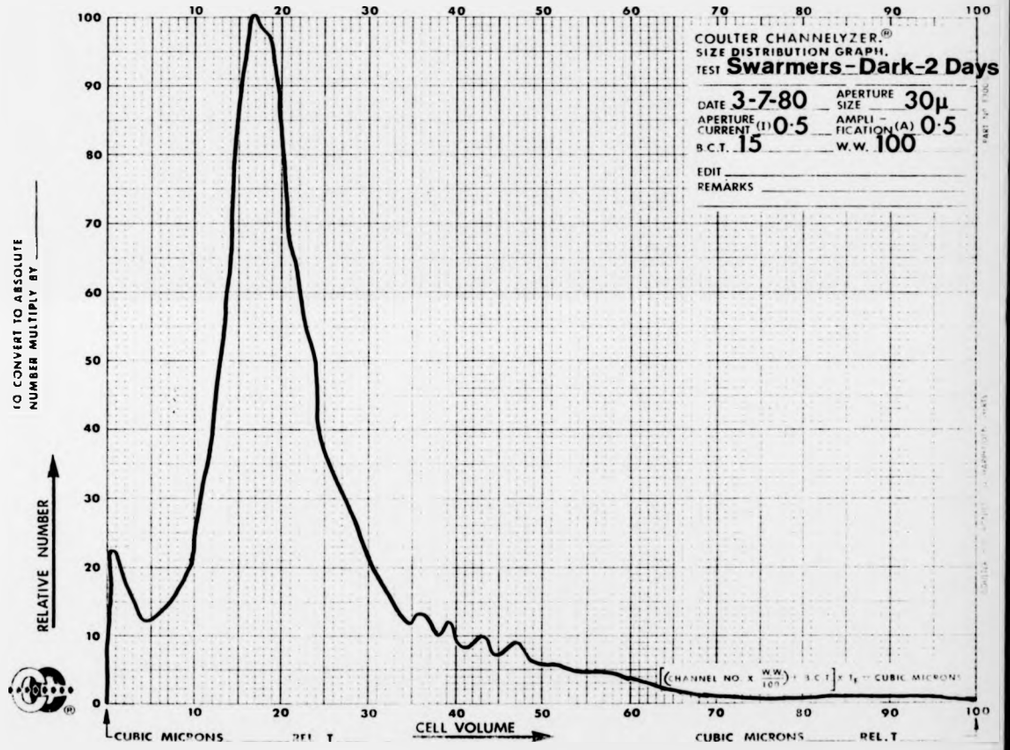
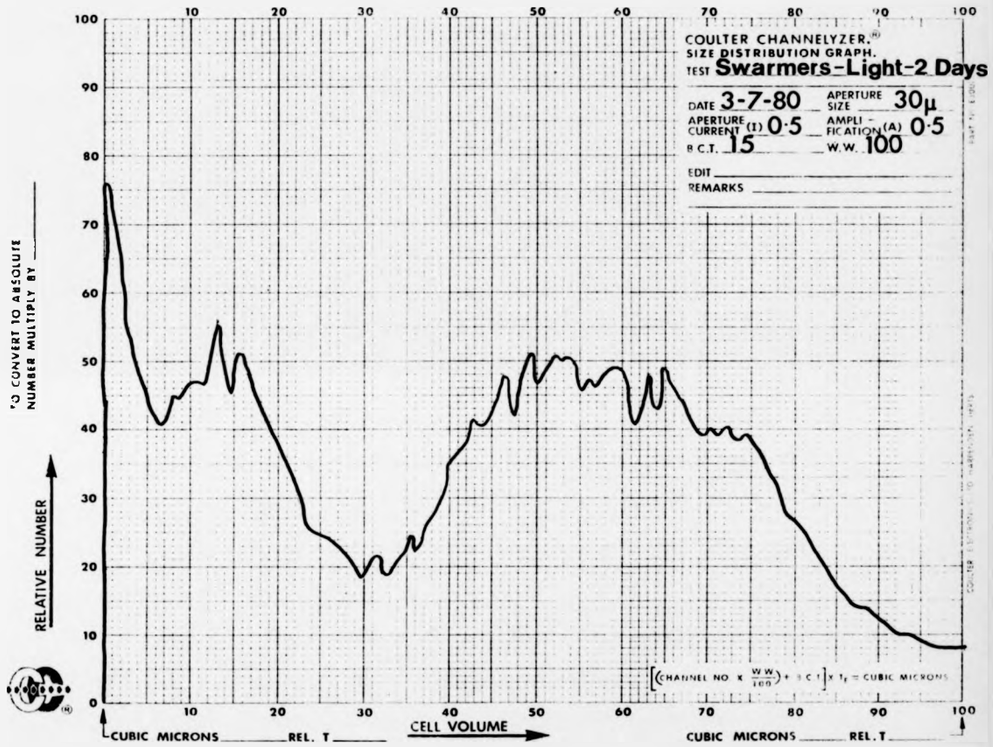


Figure 3.11

Coulter Counter size distribution profile showing synchronised swarmer cells after 2 days incubated in the light. The cells have differentiated to produce stalked cells, multicellular arrays and swarmer cells.



arrays (confirmed by microscopy). Figure 3.12 shows that label was incorporated into trichloracetic acid precipitable material both in cultures maintained in the light and in the dark. The rate of incorporation in the dark was about half that in the light. It appears that swarmer cells can incorporate radiolabel into protein although they do not develop and this is presumably accounted for by protein turnover. Figure 3.13 shows the result of a similar experiment in which 2 μCi/ml and 4 μCi/ml was added to aliquots of synchronised swarmer cells. The rate of incorporation of radiolabel was approximately proportional to the amount added in contrast to the situation found with an heterogeneous culture where the initial rate showed little variation with a change in the concentration of radiolabel. The reason for this is unclear but is probably connected with the greater number of cells present in the heterogeneous culture, 1.8×10^9 ml^{-1} compared with synchronised swarmer cells at 2×10^8 ml^{-1}, or with the different functional and physiological state of the multicellular arrays in the heterogeneous culture compared to the swarmer cells in synchronised cultures.

In these experiments with synchronised swarmer cells, 80% of the radiolabel incorporated after 24 hours was already incorporated after 6 hours so that the slowing down of incorporation was due to exhaustion of radiolabel and cannot be used as an indicator of rates of protein synthesis. In order to slow incorporation so that only a small proportion of the total radiolabel was incorporated after 6 hours varying concentrations of unlabelled L methionine were added. It was found that concentrations of 2-5 μM slowed incorporation sufficiently without reducing this to very low levels. Figure 3.14 shows that incorporation increases at 3 to 3½ hours into the synchronous

Figure 3.12 Incorporation of ^{35}S L methionine by synchronised swarmer cells under light and dark regimes.

⟜—⟜ 0.5 µCi ml^{-1} under a light regime

●—● 0.5 µCi ml^{-1} under a dark regime

Incorporation of ^{35}S L methionine was determined as described in section 2.10. Under the light regime the swarmers differentiated to produce stalked cells and daughter cells after 6 hours but under the dark regime differentiation was inhibited and the cells remained as motile swarmer cells.

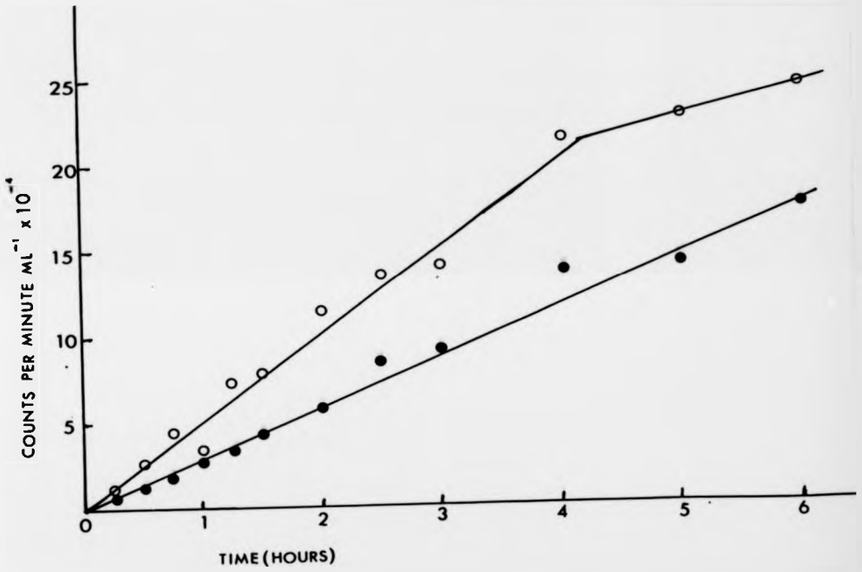


Figure 3.13 Incorporation of ^{35}S L methionine by synchronised swarmer cells under a light regime.

●—● 4 μCi ml^{-1}

⊙—⊖ 2 μCi ml^{-1}

⊡—⊡ 0.5 μCi ml^{-1}

Incorporation of ^{35}S L methionine was determined as described in section 2.10 and the initial rate was found to be approximately proportional to the concentration of radiolabel.

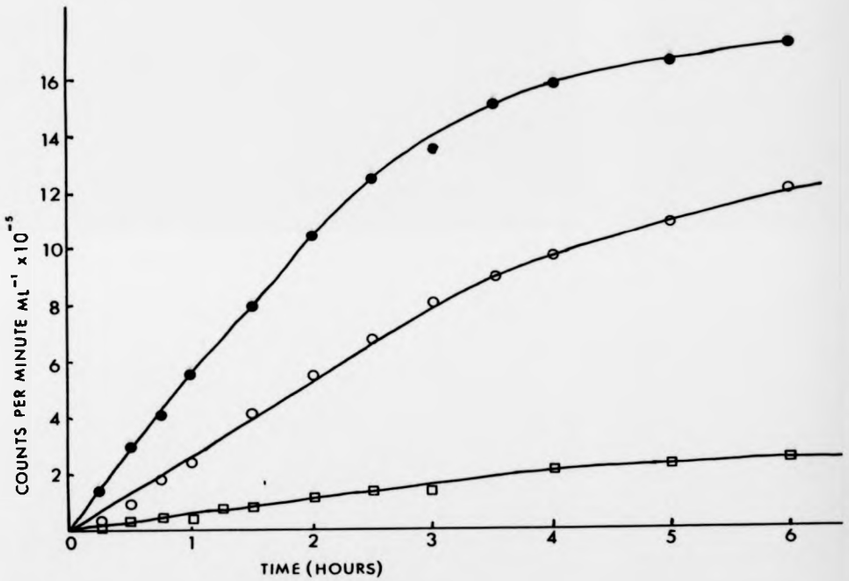
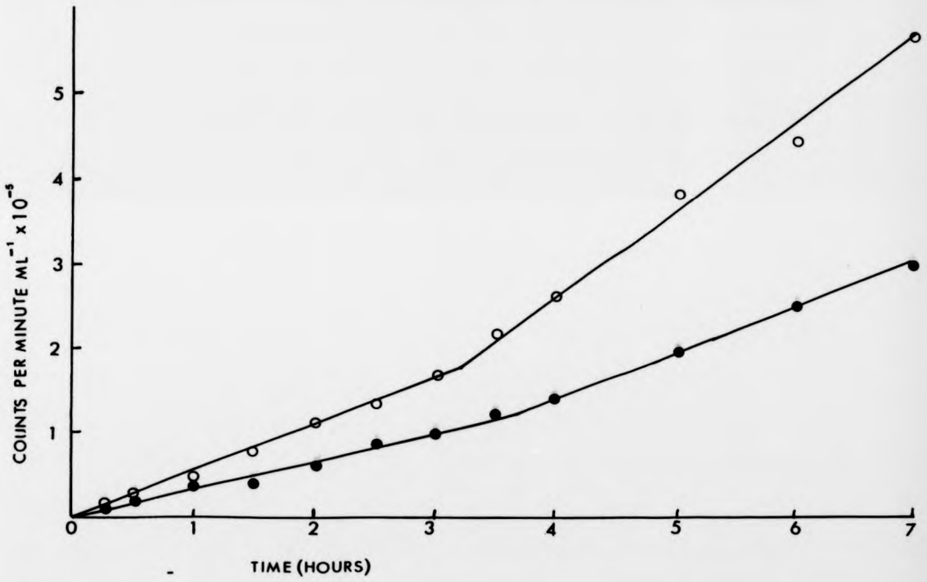


Figure 3.14 Use of the incorporation of ^{35}S L methionine to follow protein synthesis in synchronised swarmer cells.

⊖—⊖ 2 μCi ml^{-1} ^{35}S L methionine + 2 μM L methionine

●—● 2 μCi ml^{-1} ^{35}S L methionine + 5 μM L methionine

Incorporation of ^{35}S L methionine was determined as described in section 2.10 in the presence of unlabelled L methionine. The results indicate that protein synthesis increases after approximately 3.5 hours differentiation corresponding to the start of stalk synthesis.



differentiation corresponding with the start of stalk synthesis.

3.7 Longevity of Synchronised Swarmer Cells

Swarmer cells were synchronised from a 3 day old heterogeneous culture in the late exponential phase and a 200 ml sample kept under dark conditions at 30°C in a shaking water bath. Samples were taken aseptically at intervals and viable counts determined by plating onto pyruvate-malate agar and incubating under phototrophic conditions.

The strongly biphasic nature of the loss of viability (see Table 3.2 and Figure 3.15) implies the presence of two populations, the majority being relatively short lived swarmer cells with a small population of longer lived cells, consisting of about 1%, which are probably exospores (Whittenbury and Dow, 1977).

The synchronised swarmer cells remained motile as judged by light microscopy for 24 hours but after this time motility was lost. The Coulter Counter Size distribution remained typical of that for swarmer cells throughout the experiment (Figure 3.10 illustrates a synchronised swarmer cell size distribution) although after 15 days cells lysis began to occur. The small proportion of exospores needed to explain the biphasic nature of the loss of viability was not detectable by light microscopy.

Under anaerobic dark conditions swarmer cells survive at almost 100% for 24 hours then rapidly lose viability under dark conditions. This is in keeping with a role for swarmer cells in distribution and adaptation to new environments rather than long term survival, a role undertaken by the exospore (Gorlenko, 1974). The continuation of

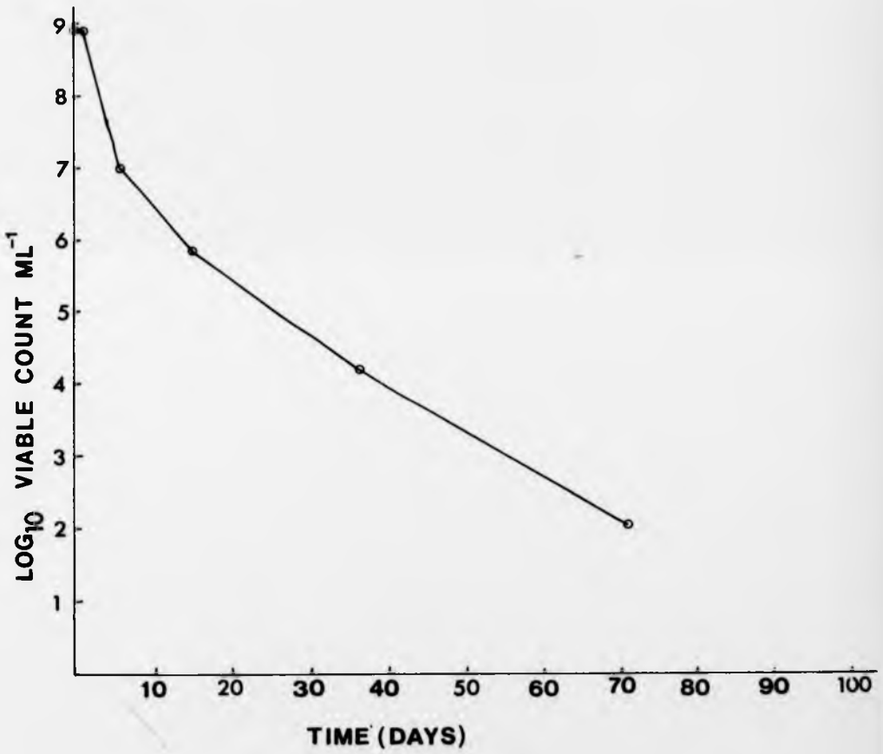
Table 3.2

Swarmer Cell Longevity

Days in Dark	Viable Count ml^{-1}	$\log_{10}$ Viable Count
0	7.5×10^8	8.88
1	6.7×10^8	8.83
6	1.0×10^7	7.0
15	6.0×10^5	5.78
36	1.6×10^4	4.20
71	1.1×10^2	2.04

Figure 3.15

Survival of synchronised swarmer cells under dark conditions at 30°C determined by viable plate counts.



protein synthesis in the swarmer cell when differentiation is inhibited by low light levels (section 3.6) shows that it is not a totally dormant resting or survival stage comparable with the endospore of Bacillus spp.

3.8 The Pattern of Proteins in Synchronised Swarmer Cells, Multicellular Arrays, and Swarmer Cells Allowed to Develop under a Light Regime

Swarmer cells were synchronised from a 3 day old 5 litre culture and harvested except for a 150 ml culture which was allowed to develop under a light regime for 14 hours. The multicellular arrays trapped in the glass wool column were washed free and harvested. Lysates were prepared as described in section 2.11 and two dimensional non-equilibrium pH gradient electrophoresis (NEPHGE) gels prepared as described in section 2.13.5, 50 µg of protein being loaded per gel. The gels were silver stained as described in section 2.14.2 and photographed. Figure 3.16 shows the pattern of proteins present in (i) synchronised swarmer cells, (ii) multicellular arrays, and (iii) swarmer cells allowed to develop for 14 hours by which time arrays of 6 cells had been produced. The protein patterns produced from newly synchronised swarmer cells and the multicellular arrays were very similar but the pattern produced from cells allowed to develop under a light regime had several differences. Some of the more obvious differences were numbered in Figure 3.16 and tabulated in Table 3.3. Some protein spots such as 1,2,5,6 and 11 were only present or much stronger in the pattern from cells allowed to develop. Conversely other protein spots such as those numbered 4,7,9 and 12 were present in

Figure 3.16

Silver stained two-dimensional NEPHGE gels showing the pattern of proteins present in (i) synchronised swarmer cells, (ii) multicellular arrays and (iii) swarmer cells allowed to develop for 14 hours.

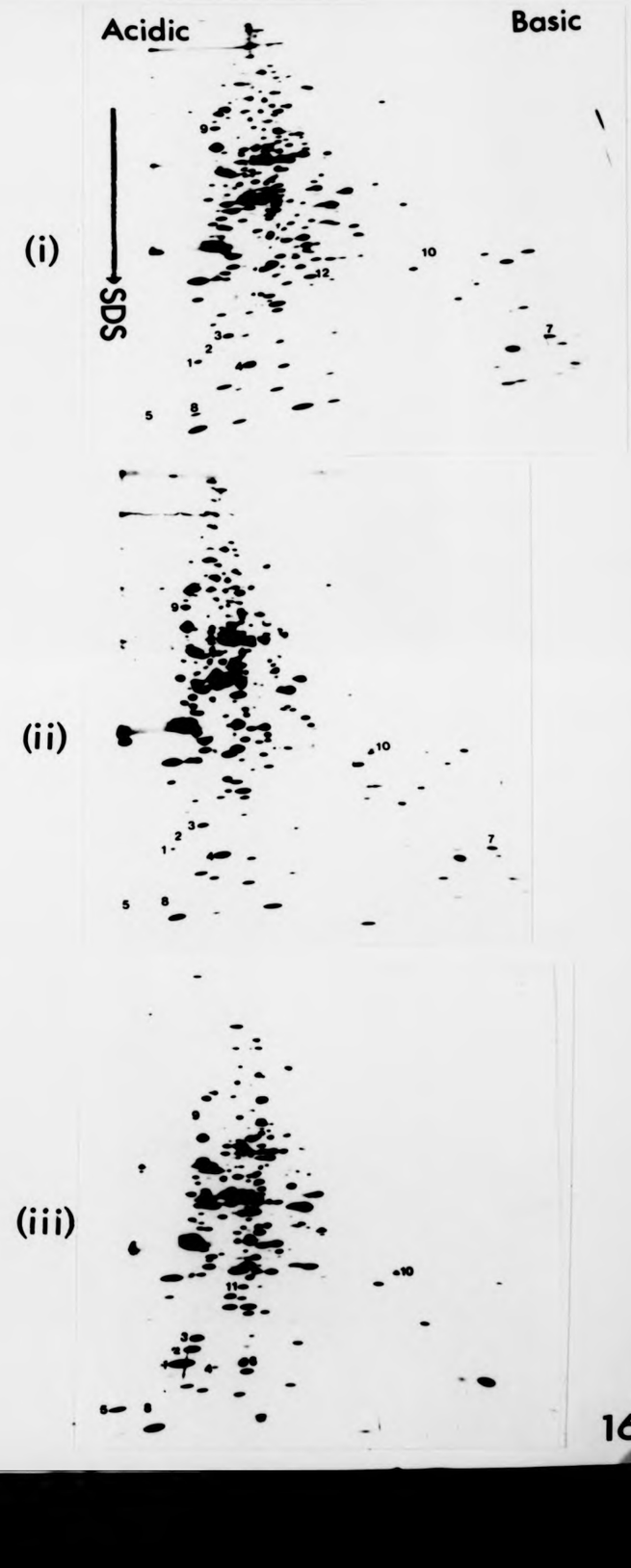


Figure 3.16

Silver stained two-dimensional NEPHGE gels showing the pattern of proteins present in (i) synchronised swarmer cells, (ii) multicellular arrays and (iii) swarmer cells allowed to develop for 14 hours.

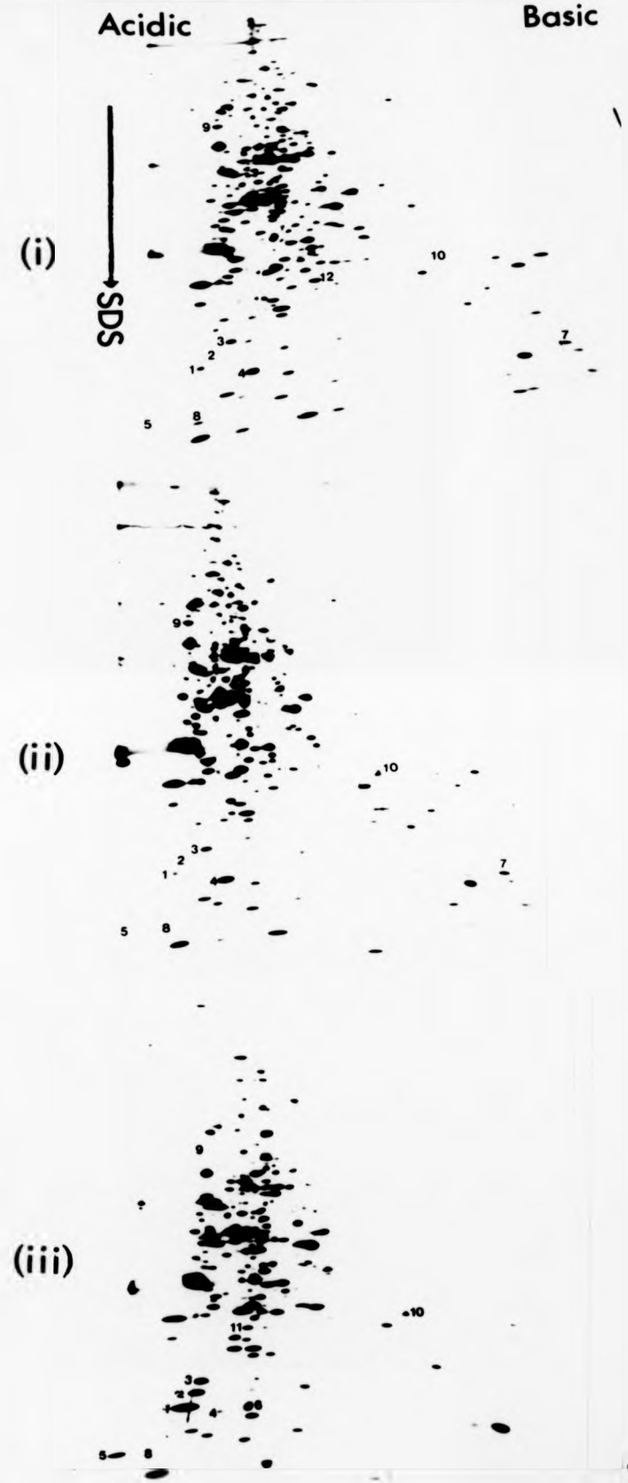


Table 3.3

Pattern of Proteins Present in Different Cell Types (discussed in section 3.8)

Protein numbered in Figure 3.16	Indication of presence in: (i) Swarmer cells	(ii) Multicellular arrays	(iii) Cells under a light regime
1	(+)	(+)	+++
2	(+)	(+)	+++
3	+	+	++
4	+	+	(+)
5	-	-	+
6	-	-	+
7	+	+	-
8	+	(+)	-
9	+	+	-
10	-	+	+
11	-	-	+
12	+	+	-

synchronised swarmer cells and multicellular arrays but absent or very faint in the pattern from cells allowed to develop for 14 hours.

The major dichotomy was between cells that had been incubated phototrophically for some time under a light regime and those from a 3 day old heterogeneous culture where the optical density of the culture had increased and light was limiting on a per cell basis. The swarmer cells and multicellular arrays separated from this culture showed very similar patterns. The sample of multicellular arrays did inevitably contain some swarmer cells trapped in the glass wool but the synchronised swarmer cells contained very few stalked cells (less than 1%) so the close resemblance in pattern was most probably real. Protein spot 8 was present in synchronised swarmer cells but very faint in the multicellular array sample and absent from the cells growing without light limitation and appeared to be a swarmer specific protein. Another exception to the major dichotomy was found in protein spot 10 which was absent from synchronised swarmer cells but found in both multicellular arrays and cells growing without light limitation may possibly be a stalk specific protein.

In summary there are several protein differences between cells growing without light limitation and those in a 3 day old culture which may be becoming light limited. There are few differences however between multicellular arrays and the swarmer cells which arise from them.

Silver stained two dimensional polyacrylamide gels provide no evidence as to the synthesis of proteins during the differentiation of swarmer cells but only show the abundance of particular proteins in the precursor swarmer cell and the differentiated stalked cells and multicellular arrays.

In some simple developmental systems such as the cell cycle of Escherichia coli or Saccharomyces cerevisiae the great majority of proteins are synthesised at each stage (Lutkenhaus et al., 1979; Elliot and McLaughlin, 1978). However in these cases morphologically different cell types are not produced. In organisms with a more complex cell cycle such as Caulobacter, Myxococcus and Dictyostelium some proteins are only synthesised in certain cell types (Sheffery and Newton, 1981; Kaiser et al., 1979; Blumberg and Lodish, 1980b). In the case of Caulobacter crescentus the flagellar proteins, flagellin A, flagellin B and hook protein are unique to the swarmer cell. Even in these cases however the majority of proteins are synthesised in all cell types. Indeed with the dimorphic yeast Candida albicans which can differentiate to produce a mycelial form or remain as a budding yeast few differences in major proteins were found between the two cell types in a two-dimensional analysis (Brummel and Soll, 1982). Again with the fungus Mucor mucedo few proteins are specific to the yeast form or to hyphae which develop in 100% nitrogen but a large number are regulated quantitatively during the morphological differentiation (Hiatt et al., 1980).

In the case of Rhodomicrobium vannielii there are few proteins that are specific to a particular cell type but as will be shown later in sections 3.9 and 3.10 many proteins are controlled quantitatively during the differentiation of the swarmer cell.

3.9 Changes in the Pattern of Protein Synthesis during Swarmer Cell Differentiation

Swarmer cells were synchronised from a 3 day old 5 litre culture and allowed to differentiate under the usual growth conditions. Aliquots were taken at intervals and incubated with 0.5 μCi ml^{-1} of ^{35}S L methionine for 10 minutes further incorporation being stopped by the addition of L methionine to 1 mM. The cells were collected by centrifugation, drop frozen in liquid nitrogen and stored at $-70^{o}C$ until required. The cells were washed with sonication buffer and broken by sonication as described previously. The soluble protein was analysed by 10-30% SDS PAGE and the gel dried and subjected to autoradiography. The autoradiograph is shown in Figure 3.17 and the stage reached in swarmer differentiation at the time of radiolabelling is shown in Table 3.4. The gel contained 100,000 cpm of ^{35}S per track. As may be seen from Figure 3.17 the pattern of protein synthesis changes markedly with time. Many bands remained approximately constant while others were synthesized more strongly at particular stages. The differences however were of degree only and no bands disappeared completely. It should be borne in mind that the separation on these SDS gels was by molecular weight only and that only about 100 bands could be resolved. This is at least an order of magnitude less than the total number of proteins synthesised in Rhodomicrobium vannielii therefore on average each band will consist of several protein species. The protein bands that vary in intensity may be divided into three groups. Firstly those synthesised strongly at the start of differentiation such as those bands labelled C, D, E, F and B which were synthesised very strongly at 30 minutes then less strongly up to 6 hours. Secondly those bands synthesised most strongly between 2

Figure 3.17

Changes in the pattern of protein synthesis during the differentiation of synchronised swarmer cells as shown on 10-30% polyacrylamide denaturing gels.

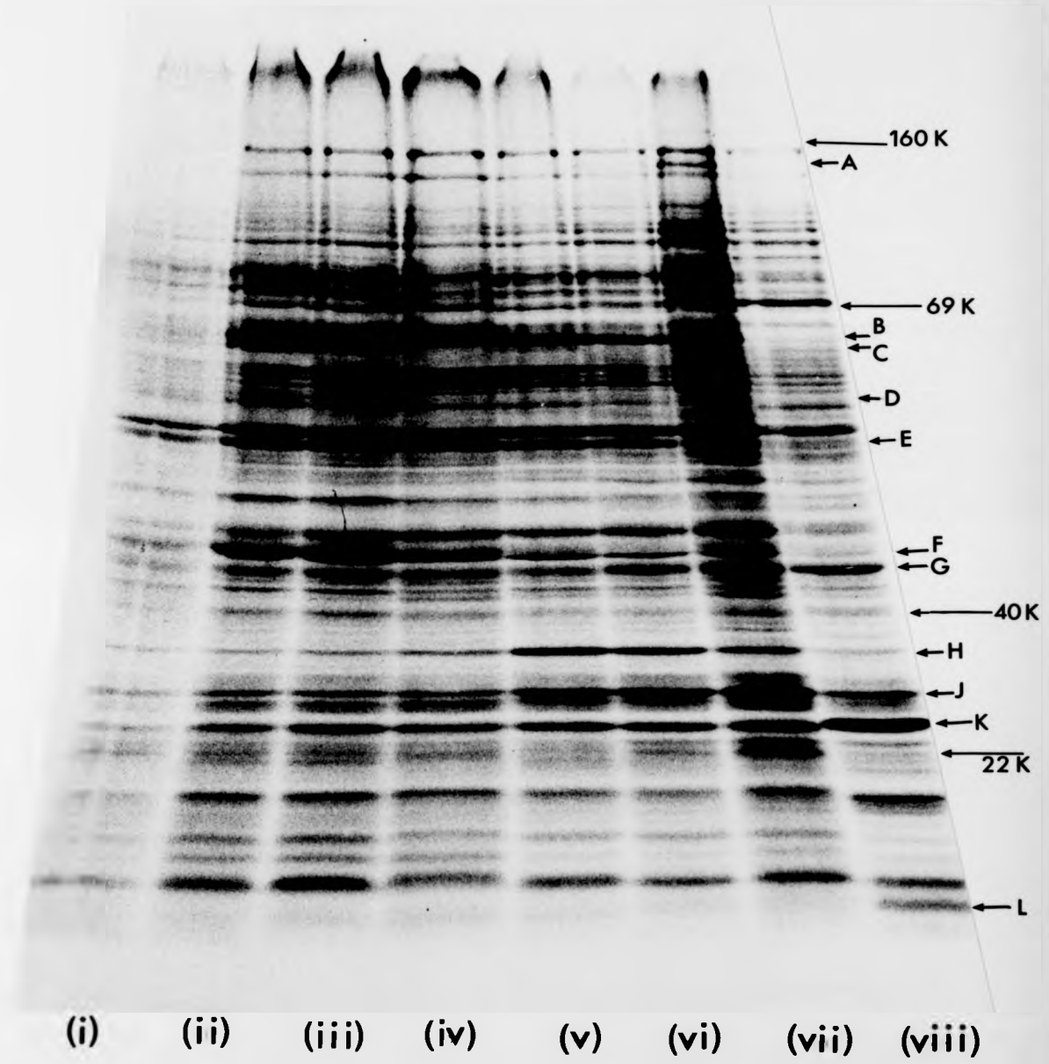


Figure 3.17

Changes in the pattern of protein synthesis during the differentiation of synchronised swarmer cells as shown on 10-30% polyacrylamide denaturing gels.

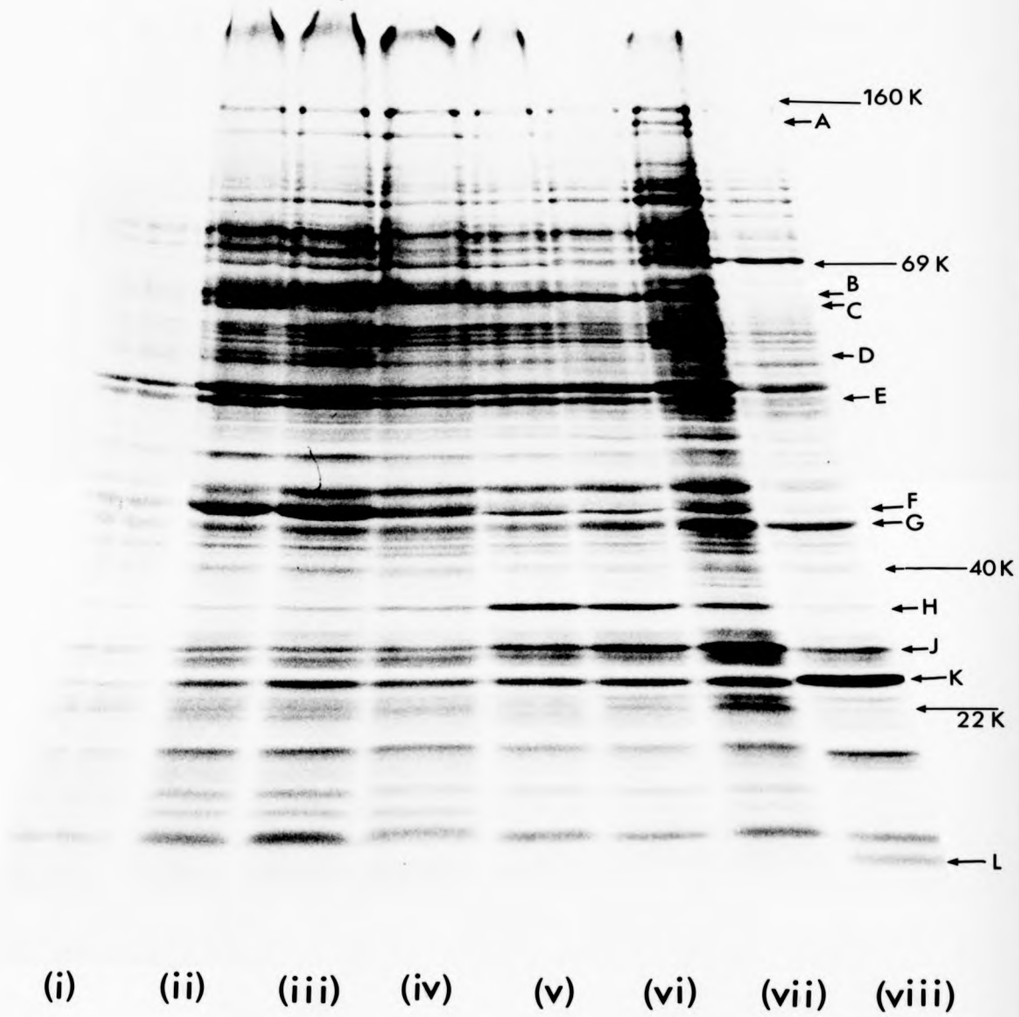


Table 3.4

Differentiation of Synchronised Swarmer Cells

Track	Time of Radiolabelling	Stage of Development
(i)	Immediately following synchronisation	Motile swarmer cells
(ii)	15 minutes	Motile swarmer cells
(iii)	30 minutes	Motile swarmer cells
(iv)	1 hour	Non-motile cells
(v)	2 hours	Cells growing stalks
(vi)	4 hours	Daughter cell synthesis begins
(vii)	6 hours	Daughter cell fully grown
(viii)	21 hours	Arrays of cells with swarmers present

hours and 6 hours into differentiation - such as bands labelled A, G, H and J. Thirdly those bands synthesised strongly after 21 hours incubation - such as bands labelled K and L. The first group's synthesis period corresponds to the period before stalk synthesis when the cells were still motile swarmers or had lost motility. The second stage corresponded to stalked and daughter cell synthesis and in the third stage multicellular arrays and new swarmer cells had been produced. It appeared therefore that stages in differentiation of synchronous swarmer cells can be correlated with quantitative changes in the synthesis of certain proteins.

3.10 Changes in the Pattern of Protein Synthesis during Differentiation Visualised by Two-Dimensional O'Farrell Gels

Swarmer cells were synchronised from a 3 day old 5 litre culture and allowed to differentiate under the usual growth conditions. Aliquots were taken at intervals and incubated wtih 0.5 $\mu Ci\ ml^{-1}$ of ^{35}S L methionine for the intervals indicated later. The cells were collected and lysates prepared as described previously. 10-30% PAGE and two-dimensional O'Farrell gels were prepared as described in section 2.13.4, 10^6 cpm being loaded per gel and autoradiographs prepared. Figure 3.18 (i), (ii), (iii) and (iv) show autoradiographs prepared from cells labelled at the following times: (i) 0 to 2 hours; (ii) 2 to 4 hours; (iii) 4 to 6 hours; (iv) 22 to 24 hours. Stalk synthesis began at 2 hours and daughter cell synthesis at 4 hours, the cycle being complete by 6 hours. After 24 hours chains of 10 to 12 cells had developed and new swarmer cells had been produced. The patterns of

Figure 3.18

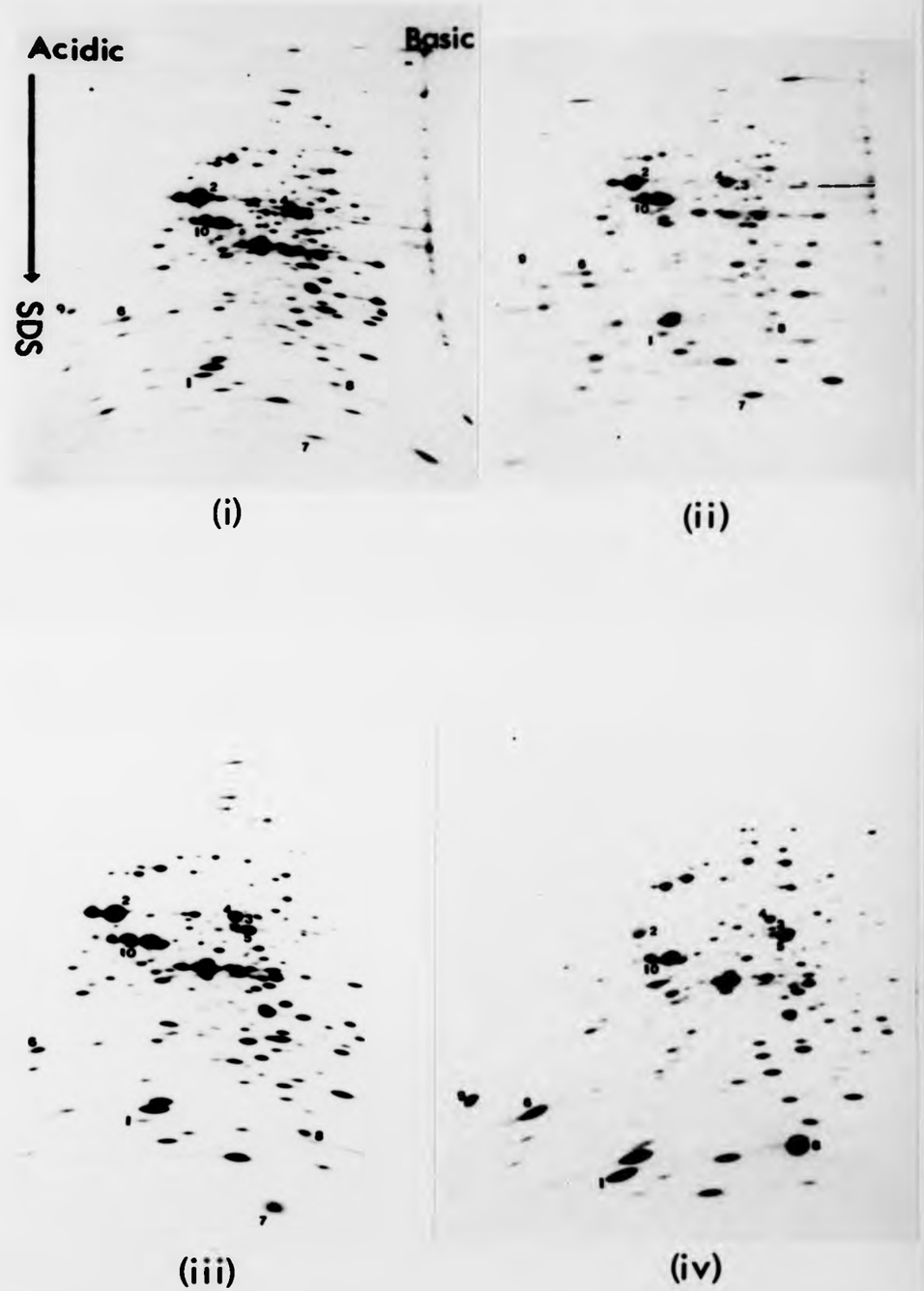
Autoradiographs of two-dimensional O'Farrell gels of proteins labelled during swarmer cell differentiation.

(i) Labelled from 0 to 2 hours

(ii) Labelled from 2 to 4 hours

(iii) Labelled from 4 to 6 hours

(iv) Labelled from 22 to 24 hours



proteins synthesized at different times were very similar especially over the first 6 hours. The pattern after 24 hours showed more changes but was overall still similar. The one-dimensional pattern is shown in Figure 3.19 showing how the pattern of changes was obscured by the superimposition of bands compared to the two-dimensional technique. A number of proteins that show marked differences in synthesis between different gels have been marked and numbered on the Figure 3.18 and are tabulated in Table 3.5. Most proteins appear to be synthesised at each stage but there are several which are synthesised mainly or entirely during certain time periods such as protein spot 3 confined to the first two hours and spots 1 and 9 only synthesised in the multicellular arrays. Some proteins such as 2, 4 and 10 were synthesised throughout differentiation to the daughter cell stage but were not seen in the multicellular arrays after 24 hours. Other proteins such as 5, 6 and 8 first appeared during stalk synthesis or daughter cell growth and were still being synthesised when multicellular arrays were present, while a protein such as number 7 was only synthesised strongly during stalk and daughter cell synthesis. In section 3.9 pulse labelling for 10 minutes with ^{35}S L methionine and analysis with one-dimensional SDS PAGE showed many quantitative changes in protein synthesis with a broad division into 3 groups. It proved to be impossible to incorporate sufficient radiolabel to permit autoradiography of two-dimensional gels so 2 hour labelling periods were used to try to take advantage of this division into 3 groups. This broad division remains evident in two-dimensional gels with the longer labelling times but the differences are much less marked than might have been expected from the data shown in section 3.9. However these differences on one-dimensional SDS PAGE are only in band intensity and no bands disappeared entirely. With two-dimensional PAGE intensity it is difficult to record a protein spot as being more than

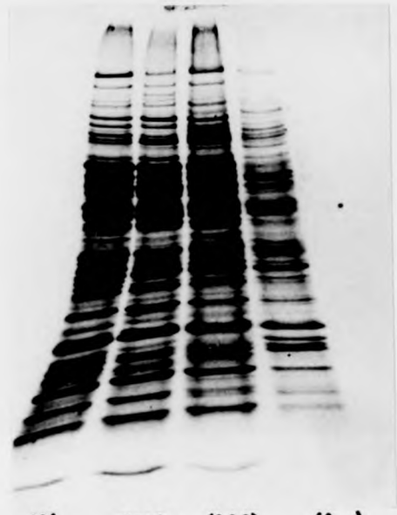
Figure 3.19 Pattern of Proteins Synthesized during Swarmer Cell Differentiation

(i) Swarmer cells - labelled from 0 to 2 hours

(ii) Stalk formation - labelled from 2 to 4 hours

(iii) Daughter cell synthesis - labelled from 4 to 6 hours

(iv) Multicellular arrays - labelled from 22 to 24 hours.



(i) (ii) (iii) (iv)

Table 3.5

Proteins Synthesised During Swarmer Cell Differentiation (discussed in section 3.10)

Protein numbered in Figure 3.18	Indication of synthesis during differentiation			
	(i) 0 to 2 hrs	(ii) 2 to 4 hrs	(iii) 4 to 6 hrs	(iv) 22 to 24 hrs
1	+	+	+	+++
2	+++	+++	+++	+
3	+++	+	+	+
4	+++	+++	+++	+
5	-	-	++	+++
6	+	+	++	+++
7	+	++	++	-
8	+	+	++	+++
9	+	(+)	-	+++
10	+++	+++	+++	+

present or absent. The long labelling time also probably allows proteins being synthesised at a low rate to become radiolabelled sufficiently to produce a spot on the autoradiograph and conversely proteins only synthesised briefly during the cell cycle may be unsufficiently labelled to become visible.

With Escherichia coli (Lutkenhaus et al., 1979) and Saccharomyces cerevisiae (Elliott and McLaughlin, 1978; Jorincz et al., 1982) most protein synthesis is not periodic and the majority are synthesised throughout the cell cycle. In the myxobacteria about one-quarter of proteins show significant changes during fruiting body formation (Kaiser et al., 1979), and in Caulobacter crescentus a number of proteins are made at defined periods in the cell cycle or in only one of the two cell types (Sheffery and Newton, 1981; Milhausen and Agabian, 1981; Ohta et al., 1982). During the fruiting body formation of Dictyostelium discoideum the initiation of synthesis of a significant number of genes occurs at the point when multicellularity is achieved prior to differentiation into spore and stalk cells (Blumberg and Lodish, 1980b). Two dimensional electrophoresis of proteins from the true slime mould Physarum polycephulum showed that most, if not all, proteins are synthesised throughout the cell cycle but that differential rates of synthesis occur at least in 30 relatively abundant proteins (Turner et al., 1981).

The results presented in sections 3.8, 3.9 and 3.10 indicate that with Rhodomicrobium vannielii also protein synthesis is periodic in the cell cycle with quantitative changes occurring during swarmer cell maturation, stalk synthesis, daughter cell synthesis and during the development of multicellular arrays. Differences are also apparent between cells growing without light limitation and late exponential phase cultures.

3.11 Protein Synthesis in Swarmer Cells Incubated under Dark and Light Regimes

Swarmer cells were synchronised from a 3 day old heterogeneous culture and 100 ml aliquots labelled with 1 μCi ml^{-1} of ^{35}S L methionine under different regimes of light and dark. Cell lysates were prepared and subjected to 10-30% SDS gel electrophoresis and autoradiographs prepared. 300,000 cpm were loaded per track. Figure 3.20 shows an autoradiograph of lysates from cells treated during labelling as follows:

(a) 6 hours light; (b) 6 hours dark; (c) 6 hours dark plus 15 mins light; (d) 6 hours dark plus 30 mins light; (e) 6 hours dark plus 1 hour light; (f) 6 hours dark plus 2 hours light.

Figure 3.20 shows one protein of M_r 11.5K synthesized strongly under a dark regime but not under light conditions. When a culture radiolabelled under a dark regime was exposed to light this protein began to be broken down after 30 minutes and was largely removed after 2 hours. This protein band was cut from the gel dissolved in 30% (v/v) hydrogen peroxide and radioactivity determined using Triton-Toluene scintillation fluid. Table 3.6 shows the counts per minute obtained from this band cut from tracks (a) to (f). The counts confirm the visual impression of Figure 3.20 that the 11.5 K protein was broken down, or at least disappeared, from the soluble fraction on exposure to a light regime. The increase after 15 minutes light was repeatable and may have been due to increased protein synthesis before breakdown was initiated. The samples (a) to (f) were analysed by the two-dimensional NEPHGE system described in section 2.13.5 with 900,000 cpm loaded per gel. The resulting autoradiographs are shown in Figures 3.21 and 3.22.

Figure 3.20 Protein Synthesis in Swarmer Cells Maintained under Dark and Light Regimes

(a) Synchronised cells radiolabelled for 6 hours in light - daughter cell fully grown.

(b) Synchronised cells radiolabelled for 6 hours in dark - swarmer cells.

(c) Synchronised cells radiolabelled for 6 hours in dark plus 15 minutes light - swarmer cells.

(d) Synchronised cells radiolabelled for 6 hours in dark plus 30 minutes light - swarmer cells.

(e) Synchronised cells radiolabelled for 6 hours in dark plus 1 hour light - non motile swarmer cells.

(f) Synchronised cells radiolabelled for 6 hours in dark plus 2 hours light - stalk formation.

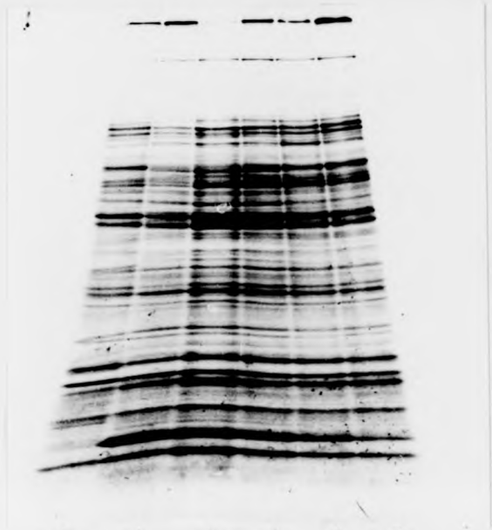


Table 3.6

Counts Incorporated into 11.5 K Protein

Track	Light Regime	c.p.m.
(a)	Light - 6 hours	1100
(b)	Dark - 6 hours	8550
(c)	Dark - 6 hours plus light - 15 minutes	11200
(d)	Dark - 6 hours plus light - 30 minutes	5400
(e)	Dark - 6 hours plus light - 1 hour	2450
(f)	Dark - 6 hours plus light - 2 hours	2100

Figure 3.21

Proteins synthesised under dark and light regimes as visualised by two dimensional electrophoresis and autoradiography.

(i) 6 hours light.

(ii) 6 hours dark, - two dimensional O'Farrell gels.

(iii) 6 hours light.

(iv) 6 hours dark - two dimensional NEPHGE gels.

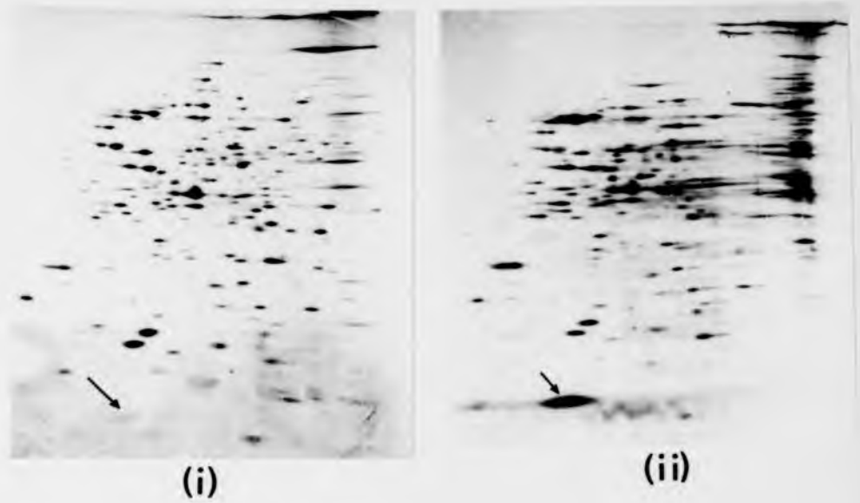


Figure 3.22

Autoradiographs of two-dimensional NEPHGE gels of proteins synthesised under various regimes of light and dark.

(i) 6 hours dark plus 15 minutes light.

(ii) 6 hours dark plus 30 minutes light.

(iii) 6 hours dark plus 1 hour light.

(iv) 6 hours dark plus 2 hours light.

(i)

(ii)

(iii)

(iv)

The 11.5 K protein is acidic with a pI of 4.5 which was later confirmed by chromatofocussing. On the two- dimensional O'Farrel system using I.E.F. in the first dimension similar results were obtained but the 11.5 K protein was more streaked than with NEPHGE gels. Gels of proteins labelled under light and dark regimes are shown in Figure 3.21 again with 900,000 cpm being loaded per gel. Cell debris from the preparation of soluble protein lysates was extracted with SDS to check whether the 11.5 K protein was sequestered in insoluble material rather than broken down. This however proved not to be the case and the protein was not found to be sequestered in insoluble material (data not shown).

Using a 5-15% non-denaturing PAGE system described in section 2.12.2 a highly radioactive band was seen at a M_r of 120 K in the lysate from swarmer cells incubated under a dark regime as shown in Figure 3.23(i). When this band was cut from the dried gel and re-analysed by SDS PAGE as described in section 2.13.3 the autoradiograph (Figure 3.23(ii)) confirmed that the 11.5 K protein was contained in the 120 K band in the non-denaturing gel. It appeared therefore that the protein was part of a larger complex in the un-denatured form.

The tracks (a) to (e) in the autoradiograph (Figure 3.24) have 100,000 cpm per track of cell lysates from swarmer cells incubated with ^{35}S L methionine as follows:

(a) 6 hour dark and anaerobic;

(b) 6 hours light and aerobic;

(c) 6 hours dark and anaerobic plus 2 hours dark and aerobic;

(d) 6 hours dark and aerobic;

(e) 6 hours dark and aerobic plus 2 hours light and aerobic.

Figure 3.24 shows that the 11.5 K protein was synthesized to a large extent only under a dark, anaerobic regime - track (a) and not

Figure 3.23 Autoradiographs of PAGE Gels.

(i) Non-denaturing polyacrylamide gel of protein labelled under a dark regime (tracks (a) and (b)) and under a light regime (track (c)).

(ii) Re-electrophoresis of arrowed band cut from gel in (i) on a denaturing 10-30% polyacrylamide gel shown in track (a) while a lysate labelled under a dark regime is shown in track (b). Shows that the native M_r of the 11.5K protein is approximately 120K.

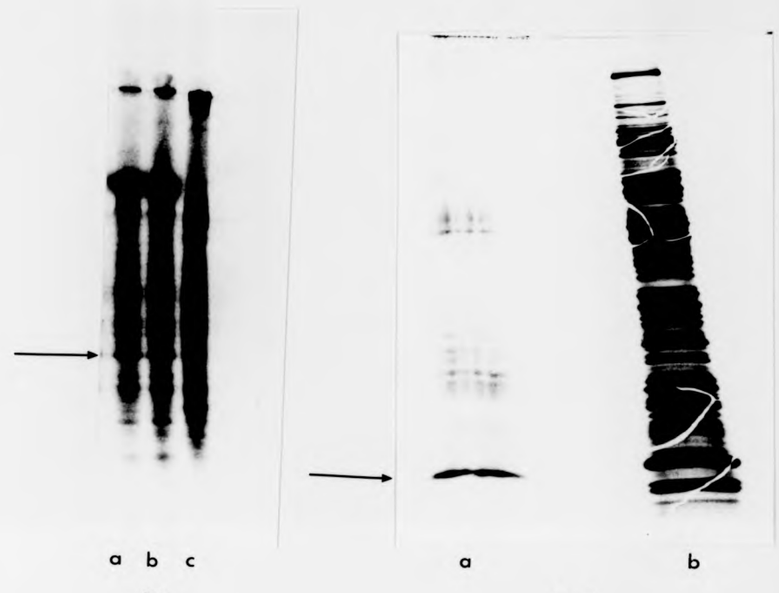


Figure 3.24

Autoradiograph of 10-30% polyacrylamide denaturing gel of proteins labelled in swarmer cells with ^{35}S L methionine under various regimes of light and dark and aerobicity.

(a) 6 hours dark and anaerobic.

(b) 6 hours light and anaerobic.

(c) 6 hours dark and anaerobic plus 2 hours dark and aerobic.

(d) 6 hours dark and aerobic.

(e) 6 hours dark and aerobic plus 2 hours light and aerobic.

under a dark aerobic regime - (d), where the 11.5 K band was just visible. Track (c) shows the effect of 2 hours of aerobic conditions after 6 hours of dark anaerobic conditions. The 11.5 K band was noticeably reduced by a 2 hour exposure to air compared with track (a). The 11.5 K protein was degraded under light or aerobic conditions and it may have been that breakdown was controlled in some way by energy levels in the swarmer cell.

Isolation of membranes and ribosomes from swarmer cells labelled with ^{35}S L methionine under dark and light regimes followed by SDS PAGE showed that the 11.5 K protein was not a membrane protein or ribosomal (data not shown) and it appears to be a soluble protein.

The function of the 11.5 K protein is as yet unknown but its synthesis correlates with inhibition of differentiation in the light limited swarmer cell and its degradation coincides with the relief of that inhibition and consequent differentiation to produce a stalked reproductive cell.

In Escherichia coli an 11K protein has been found which is degraded by the lon (capR) ATP hydrolysis-dependent protease and which is postulated to be involved in the inhibition of cell division (Schoemaker et al., 1982), but is apparently not part of the SOS response. The lon (capR) protease is also involved in the degradation of the 18 K sul A protein which is a UV-inducible division inhibitor involved in the SOS response to chromosome damage by radiation (Mizusawa and Gottesman, 1983).

Whether the 11.5 K protein found in R. vannielii swarmer cells is directly involved in the inhibition of cell division in some way, such as regulation of the transcription of operons, is at present unknown.

3.12 Comparison of the Proteins Synthesised under Dark and Light Regimes by Double Labelling and Two-Dimensional Electrophoresis

A 3 day old heterogeneous culture was synchronised and 100 ml aliquots radiolabelled under regimes of light and dark with either ^{35}S L methionine or ^{3}H L methionine.

The samples were radiolabelled under the following regimes:

1) 6 hours in the dark with 2.5 µCi ml^{-1} of ^{3}H L methionine
2) 6 hours in the light with 1 µCi ml^{-1} of ^{35}S L methionine
3) 6 hours in the dark with 1 µCi ml^{-1} of ^{35}S L methionine
4) 6 hours in the light with 2.5 µCi ml^{-1} of ^{3}H L methionine

Synchronous cultures incubated under dark conditions remained as swarmer cells which regained motility in the light while cultures incubated in the light developed to produce daughter cells.

Cell lysates were prepared from these samples and two-dimensional O'Farrell and NEPHGE gels prepared using pairs of samples as described in sections 2.13.4, 2.13.5 and 2.17. Fluorographs and autoradiogrpahs were prepared as described in sections 2.16 and 2.17. Fluorographs were also prepared a year later when the ^{35}S had decayed to levels at which it could not be detected after the passage of four half lives. The protein patterns of ^{35}S and ^{3}H labelled proteins were compared photographically as described in section 2.17. Figure 3.25 shows a fluorograph and an autoradiograph of a two-dimensional O'Farrell gel on which was loaded 600,000 dpm of sample (1) plus 200,000 dpm of sample (2). So in this case proteins synthesised under a dark regime were labelled with ^{3}H L methionine and proteins synthesised under a light regime were labelled with ^{35}S L methionine. The fluorograph in Figure

3.25(i) shows proteins labelled under both dark and light regimes while the autoradiograph in Figure 3.25(ii) shows proteins labelled under the dark regime only. The only obvious difference is the presence of an 11.5 K protein in the fluorograph (arrowed) missing from the autoradiograph. This demonstrates that ^{3}H labelled proteins do not expose the autoradiograph under the conditions outlined in 2.17.

Figure 3.26 shows fluorographs and autoradiographs of a two-dimensional NEPHGE gel which was loaded with 200,000 dpm of sample (3) and 600,000 dpm of sample (4). In this case proteins synthesised under a dark regime were labelled with ^{35}S L methionine and proteins synthesised under a light regime with ^{3}H L methionine. The fluorograph in Figure 3.26(i) shows proteins labelled both under the dark and light regimes, and the autoradiograph in Figure 3.26(iii) shows only proteins labelled under the dark regime. The fluorograph in Figure 3.26(ii) was taken a year later and should show only proteins labelled with ^{3}H L methionine if the ^{35}S had decayed to sufficiently low levels to remain undetected. That this was the case is shown by the fact that the 11.5 K protein present in Figures 3.26(i) and 3.26(iii) is absent. So Figure 3.26(ii) shows the protein pattern obtained under a light regime and Figure 3.26(iii) the pattern obtained under a dark regime. These patterns were compared photographically as described in section 2.17 and the results are shown in Figures 3.27 and 3.28. In Figure 3.27 the proteins specific to the light regime are shown as black spots while in Figure 3.28 the proteins specific to the dark regime are shown as black spots. Very few proteins are specific to the dark regime with the 11.5 K protein being the most obvious, while in the order of 25 proteins are seen to be specific to the light regime. Most proteins are however synthesised under both regimes and may be regarded as "house-keeping"

3.25(i) shows proteins labelled under both dark and light regimes while the autoradiograph in Figure 3.25(ii) shows proteins labelled under the dark regime only. The only obvious difference is the presence of an 11.5 K protein in the fluorograph (arrowed) missing from the autoradiograph. This demonstrates that ^{3}H labelled proteins do not expose the autoradiograph under the conditions outlined in 2.17.

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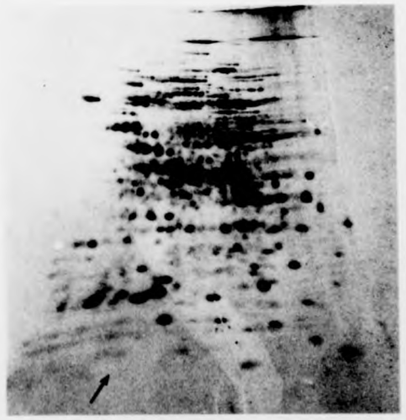
Figure 3.25

Two dimensional O'Farrell gels of proteins labelled under dark and light regimes with ^{3}H L methionine and ^{35}S L methionine repectively in swarmer cells.

(i) Fluorograph showing proteins labelled both with ^{3}H and ^{35}S L methionine under both dark and light regimes

(ii) Autoradiograph showing proteins labelled with ^{35}S L methionine only. This shows the pattern of protein synthesis under light conditions.

(i)



(ii)

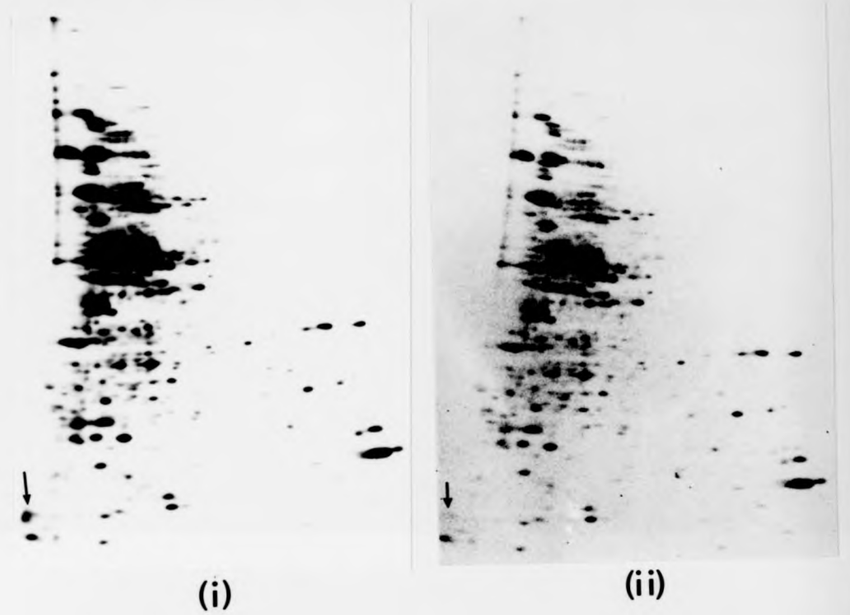
Figure 3.26

Two dimensional NEPHGE gels labelled under dark and light regimes with ^{35}S L methionine and ^{3}H L methionine respectively in swarmer cells.

(i) Fluorograph showing proteins labelled under both regimes.

(ii) Fluorograph after 1 year showing proteins labelled under light conditions.

(iii) Autoradiograph showing proteins labelled under dark conditions.



(i) (ii)

(iii)

Figure 3.27

Photographic comparison of protein patterns in Figure 3.26(ii) and (iii). Proteins only synthesised during a light regime are represented as black spots while proteins synthesised under a dark regime or under both conditions are represented as white spots. Examples of proteins only synthesised during a light regime are numbered from 1 to 10 and these may be specific to the developmental sequence or be involved in photosynthesis.

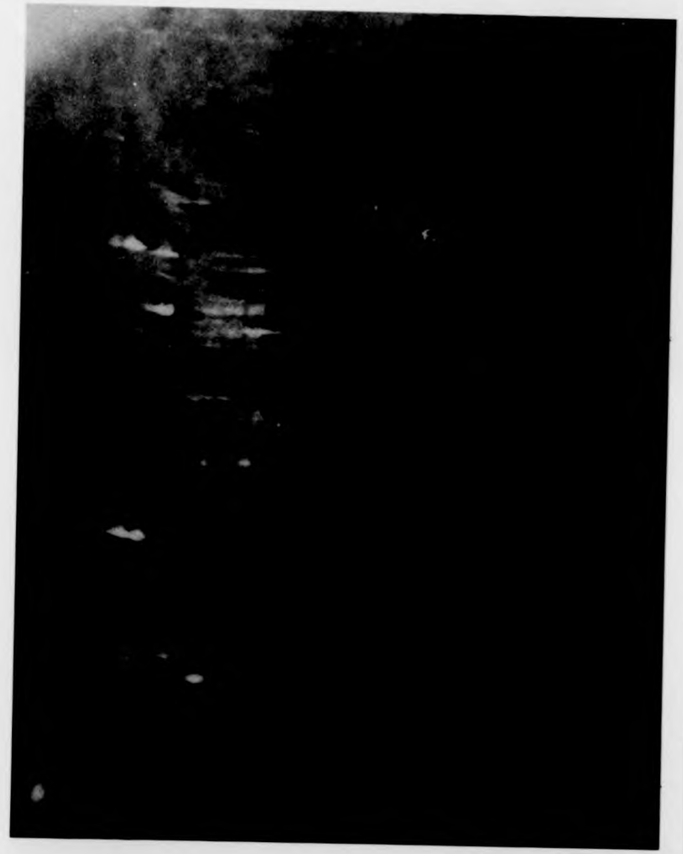
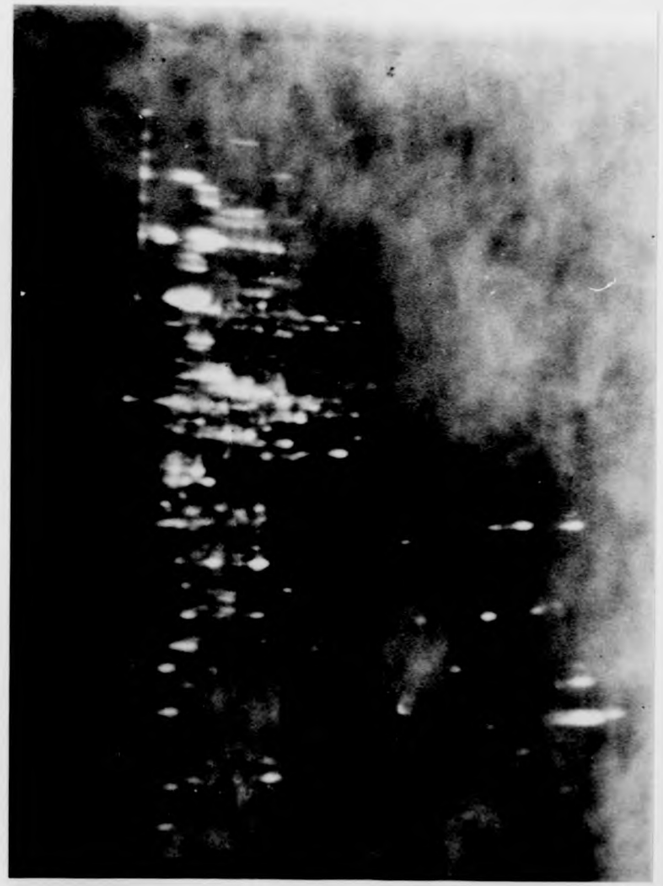


Figure 3.27

Photographic comparison of protein patterns in Figure 3.26(ii) and (iii). Proteins only synthesised during a light regime are represented as black spots while proteins synthesised under a dark regime or under both conditions are represented as white spots. Examples of proteins only synthesised during a light regime are numbered from 1 to 10 and these may be specific to the developmental sequence or be involved in photosynthesis.

Figure 3.28

Photographic comparison of protein patterns in Figure 3.26(ii) and (iii). Proteins only synthesised during a dark regime are represented as black spots while proteins synthesised under a light regime or under both conditions are represented as white spots. The proteins numbered 1,2,3 are only synthesised under a dark regime and may be involved in the maintenance of the inhibited state in the swarmer cell.



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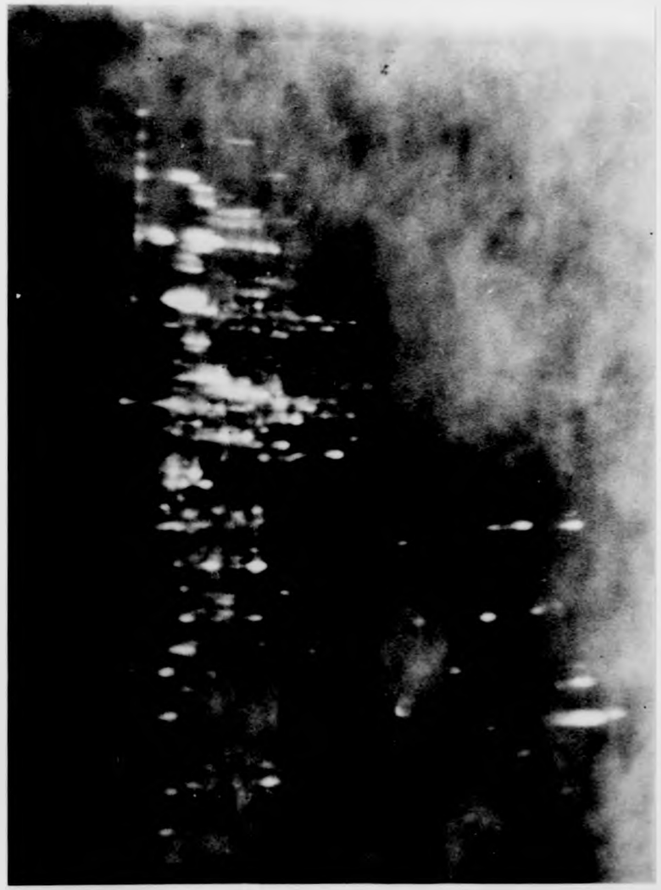
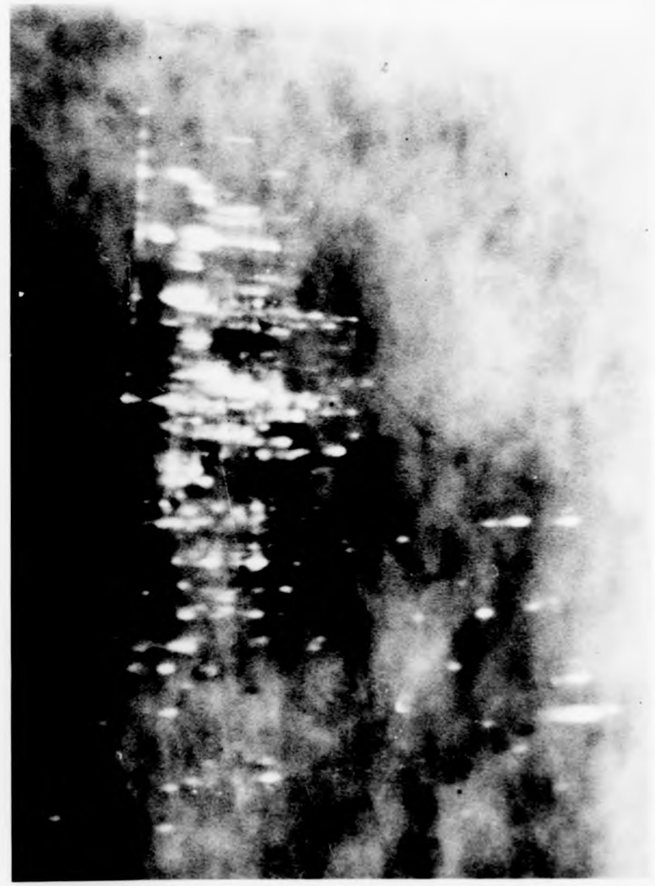


Figure 3.28

Photographic comparison of protein patterns in Figure 3.26(ii) and (iii). Proteins only synthesised during a dark regime are represented as black spots while proteins synthesised under a light regime or under both conditions are represented as white spots. The proteins numbered 1,2,3 are only synthesised under a dark regime and may be involved in the maintenance of the inhibited state in the swarmer cell.



proteins always needed under any stage of growth. Some of the proteins specific to the light regime may be specific to the developmental sequence but there are other possibilities such as involvement in photosynthesis. The function of the proteins specific to the dark regime is unknown but an involvment in the inhibition of development is one possibility.

3.13 Isolation of the 11.5 K Protein for the Preparation of Antiserum

A lysate from swarmer cells labelled under a dark regime for 6 hours with ^{35}S L methionine at 1 $\mu Ci/ml^{-1}$ was analysed together with an unlabelled cell lysate from swarmer cells subjected to the same regime by 10-30% SDS PAGE. 100 µg of total protein plus 100,000 cpm of ^{35}S was loaded in each of 12 tracks on the gel. Following electrophoresis the gel was covered with cling-film and autoradiographed on the glass front plate. The developed autoradiograph was used to provide a template for the removal of the 11.5 K protein band from the gel and this autoradiograph is shown in Figure 3.29. The excised gel was laid on top of a support gel and the protein electrophoresed upwards through a stacking gel and collected in a layer of glycerol as described in the section 2.18. This was used to raise rabbit antiserum as previously described (section 2.19).

3.14 Immunoprecipitation of the 11.5 K Protein

Lysates were prepared from swarmer cells radiolabelled with ^{35}S L

Figure 3.29

Autoradiograph of 10-30% polyacrylamide gel used to purify the 11.5K protein for raising antisera.

methionine under various regimes of dark and light as described in section 3.11. These were immunoprecipitated with immune serum as described in section 2.20 and the precipitates analysed by 10-30% (w/v) SDS PAGE followed by autoradiography. Figure 3.30 shows the resulting autoradiograph with immunoprecipitates from 500,000 cpm in the total protein loaded per track. Tracks (a) to (f) were lysates from synchronised cultures subjected to the following regimes:

a) 6 hours dark;

b) 6 hours dark plus 15 minutes light;

c) 6 hours dark plus 30 minutes light;

d) 6 hours dark plus 1 hour light;

e) 6 hours dark plus 2 hours light;

f) 6 hours light.

As can be seen from Figure 3.30 the immunoprecipitate contains only one strongly radioactive band at 11.5 K not seen in lysates from a light regime and broken down under regimes with increasing light. This provides confirmation that antibodies have been raised to the 11.5 K protein. Only two other faint bands can be seen on the autoradiograph at 23 K M_r and 66 K M_r which may be multimers of the 11.5 K protein. Non-denaturing gel electrophoresis suggested that the 11.5 K protein is part of a complex with a M_r of 120 K so it is possible that the extra bands in the Figure 3.30 represent other proteins in this complex.

3.15 ATP Levels in Swarmer and Differentiating Cells

Swarmer cells were synchronised from a 3 day old heterogeneous culture in the late exponential phase and 160 ml aliquots subjected to

Figure 3.30

Immunoprecipitation of the 11.5 K protein from ^{35}S L methionine labelled cell lysates under various regimes of light and dark analysed by 10-30% (w/v) polyacrylamide denaturing gel electrophoresis.

(a) 6 hours dark

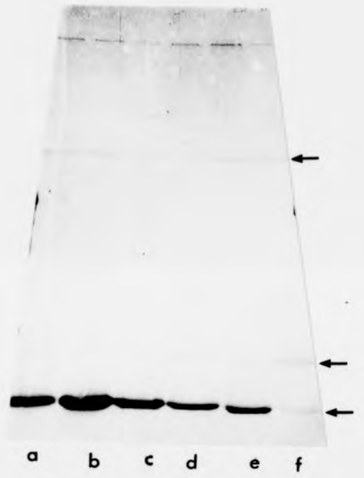
(b) 6 hours dark plus 15 minutes light

(c) 6 hours dark plus 30 minutes light

(d) 6 hours dark plus 1 hour light

(e) 6 hours dark plus 2 hours light

(f) 6 hours light.



different regimes of light and dark as follows: (a) 6 hours light; (b) 6 hours dark; (c) 6 hours dark plus 15 minutes light; (d) 6 hours dark plus 30 minutes light; (e) 6 hours dark plus 1 hour light; (f) 6 hours dark plus 1½ hours light; (g) 6 hours dark plus 2 hours light. ATP levels were measured using the luciferase system described in section 2.25. Cells were counted using the Coulter Counter. The ATP content of the cells is shown in Table 3.7 and Figure 3.31. The cell count was 1.2 x 10^8 ml^{-1}.

The ATP level in an inhibited swarmer cell rises rapidly when light is provided but peaks after about 30 minutes and declines to approximately half the peak height after 2 hours. Stalk synthesis begins at about 2 hours followed by the initiation of DNA synthesis.

During the swarmer cell differentiation of Hyphomicrobium neptunium ATP levels rose during the initial swarmer cell maturation then fell during stalk formation and DNA synthesis which may be attributable to the synthesis of purines and pyrimidines needing an input of ATP (Emala and Weiner, 1983). Both Hyphomicrobium and Rhodomicrobium have non-growing motile swarmer cell stages and maintain relatively high ATP levels compared to the maximum reached in the cell cycle probably to maintain motility.

3.16 Assay of Synthesis of the 11.5 K Protein

Swarmer cells were synchronised from a 3 day old, late exponential phase culture and 100 ml aliquots subjected to different regimes of light and dark, and ^{35}S L methionine at 1 µCi/ml added as follows: (a) 6 hours dark; (b) 6 hours dark plus 15 minutes light; (c) 6 hours

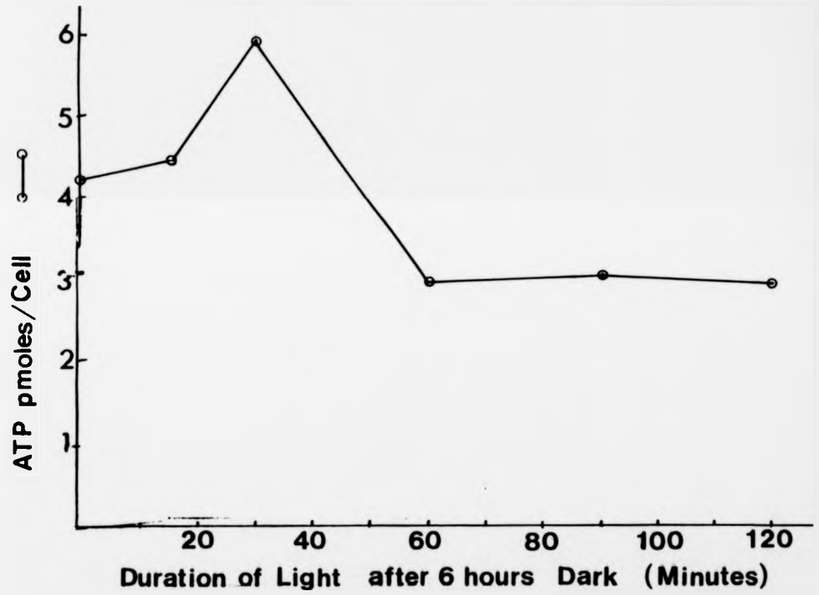
Table 3.7

ATP Levels in Differentiating Swarmer Cells

Sample	Light Regime	ATP (μmoles cell^{-1})
(a)	6 hours light	1.29×10^{-7}
(b)	6 hours dark	4.27×10^{-7}
(c)	6 hours dark plus 15 mins light	4.49×10^{-7}
(d)	6 hours dark plus 30 mins light	5.95×10^{-7}
(e)	6 hours dark plus 1 hour light	3.02×10^{-7}
(f)	6 hours dark plus 1½ hours light	3.11×10^{-7}
(g)	6 hours dark plus 2 hours light	3.03×10^{-7}

Figure 3.31

ATP levels in swarmer cells after various regimes of light following 6 hours under dark conditions. ATP levels were measured using the luciferase system described in section 2.26.



dark plus 30 minutes light; (d) 6 hours dark plus 1 hour light; (e) 6 hours dark plus 2 hours light.

Samples (a) to (e) were labelled for the whole of these periods. Samples (f) to (l) were pulse-labelled as follows: (f) 6 hours dark then labelled for 10 minutes in the dark; (g) 6 hours dark then labelled for 10 minutes in the light; (h) 6 hours dark then 10 minutes light then labelled from 10 to 20 minutes in the light; (i) 6 hours dark then 20 minutes light then labelled from 20 to 30 minutes in the light; (j) 6 hours dark then 30 minutes light then labelled from 30 to 40 minutes in the light; (k) 6 hours dark then 40 minutes light then labelled from 40 to 50 minutes in the light; (l) 6 hours dark then 110 minutes light then labelled from 110 to 130 minutes in the light.

In each case incorporation was stopped by the addition of L methionine to 1 mM. Cell lysates were prepared and 50 µl aliquots of each sample assayed for total incorporation of ^{35}S into protein by TCA precipitation and incorporation into the 11.5 K protein by immunoprecipitation, and scintillation counting. The results in terms of counts incorporated into the 11.5 K protein per 10^6 counts incorporated into total protein is shown in Table 3.8.

Figure 3.32 displays these values in graph form together with ATP levels calculated in section 3.15. Total incorporation of label into the 11.5 K protein was maximal after 15 minutes light then fell rapidly. The 10 minute pulses showed that the rate of synthesis of the 11.5 K protein rose to reach a maximum after 25 minutes exposure to light. The increase in total incorporation after 15 minutes light correlates closely with the total synthesis during this time calculated from the area under the rate of synthesis graph. The decline after 15 minutes light was presumably able to degradation of the 11.5 K protein as

Table 3.8

Counts Incorporated into the 11.5 K Protein

Sample	Immunoppt/10^6 cpm total	Immunoppt/10^6 cpm/min
(a)	28,500	
(b)	37,330	
(c)	18,000	
(d)	8,165	
(e)	7,000	
(f)	5,000	500
(g)	6,170	617
(h)	7,220	722
(i)	14,810	1,481
(j)	8,520	852
(k)	5,290	529
(l)	5,300	530

Figure 3.32

ATP levels in swarmer cells after various regimes of light and dark conditions, the incorporation of ^{35}S L methionine into the 11.5 K protein and its synthesis and degradation rates under these conditions.

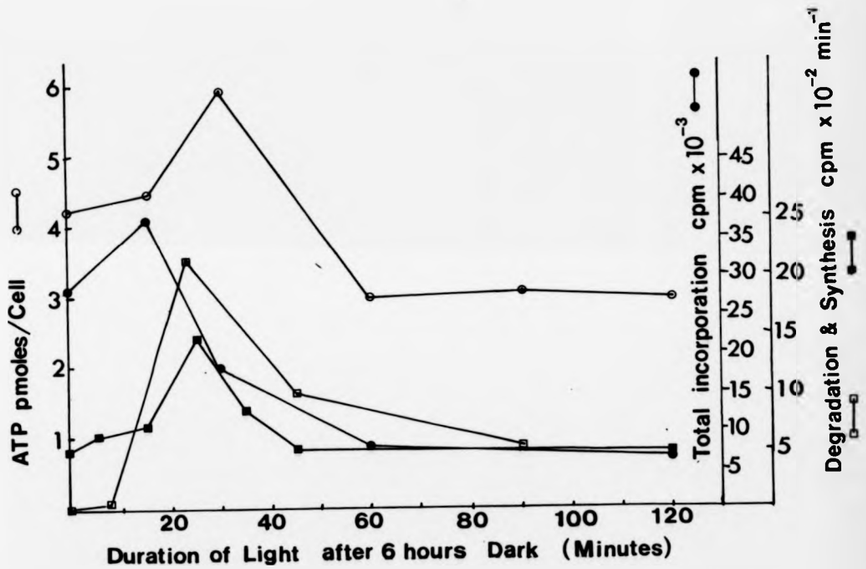


Table 3.9

Synthesis and Degradation of the 11.5 K Protein

Exposure to Light (Minutes)	Total Synthesis CPM/10^6 total	Total Incorporation CPM/10^6 total	Total Degradation CPM/10^6 total	Degradation Rate CPM/10^6 total/min
0	-	28,500	-	-
15	9,488	37,330	658	44
30	22,508	18,000	33,008	2,157
60	42,378	8,165	62,713	990
120	74,118	7,000	95,618	548

synthesis was still occurring. The rate of degradation may be calculated from the total synthesis and the total incorporation (Table 3.9 and Figure 3.32).

The rate of degradation reaches a maximum at a similar time to the rate of synthesis so it appears that the amount of 11.5 K protein present is a balance between these factors with the protein accumulating under a dark regime because of lower degradation. It should be noted that the above analysis assumes zero degradation under a dark regime so the degradation calculated is an excess value under a light regime.

Under a light regime a reduction in synthesis and continued degradation reduces the amount of the 11.5 K protein to the low level seen in cultures incubated in the light. Both synthesis and degradation are positively correlated with the ATP levels in the cell reaching maxima at similar times after exposure to light and this could be the factor controlling the amount of the 11.5 K protein in the cell.

In Escherichia coli abnormal proteins such as those produced by mistranslation are rapidly degraded in an energy dependent process stimulated by ATP (Parker, 1981; Murakami et al., 1979). Putative cell division inhibition proteins in E. coli are also degraded in an energy dependent process by the lon(capR) ATP hydrolysis-dependent protease as discussed in section 3.11 (Mizusawa and Gottesman, 1983; Schoemaker et al., 1982).

3.17 Conclusions and Potential for Future Research

The cell cycle in eukaryotes and prokaryotes is essentially very similar. In eukaryotes the cell cycle may be divided into phases. DNA

is synthesised in the "S" phase which is followed by the "G2" phase lacking DNA synthesis before mitosis and cell division. Following the cell division a further phase, "G1", lacking DNA synthesis intervenes before the start of the "S" phase. Non-dividing cells are usually arrested within the "G1" phase in what may be termed a "GO" phase.

The division cycle of Escherichia coli, commonly regarded as the type prokaryote, may at generation times up to 60 minutes be divided into a C period of DNA synthesis and then a D period before cell division (Helmstetter and Cooper, 1968; Donachie et al., 1976). At longer generation times the C period approximates to 2/3 of the generation time but the D period remains roughly constant at 20 minutes (Davern, 1979), and a variable I period follows cell division which is equivalent to the eukaryotic "G1" phase (Helmstetter et al., 1979).

In budding bacteria, such as Caulobacter crescentus, the cell cycle leads to the production of two cell types, a motile swarmer cell and a stalked mother cell. The swarmer cell has an obligatory maturation period before DNA synthesis begins which may be correlated with the eukaryotic "G1" phase and the prokaryotic I period. The stalked mother cell does not need such an I period and can initiate DNA synthesis immediately following division (Osley and Newton, 1980).

The swarmer cell of Rhodomicrobium vannielii exhibits an I period before initiation of DNA synthesis (section 3.2). However protein and RNA synthesis occur and it is probable that the synthesis and assembly of DNA initiation components takes place during this period.

During the growth of a heterogeneous culture the proportion of swarmer cells increases in the late exponential phase (section 3.4) and as is shown in the Appendix (section 4.2) these changes can be modelled by assuming that swarmer cell differentiation is inhibited by rising

optical density due to increasing cell number. This provides evidence that the length of the I period in R. vannielii is controlled by the availability of light probably acting at the level of energy.

Coulter Counter size distribution profiles of swarmer cells incubated in the dark show that they remain inhibited and do not differentiate (section 3.6). Some protein synthesis occurs under dark incubation but rRNA synthesis does not. This inhibited state can be regarded as equivalent to the eukaryotic "GO" phase in which cells not about to divide are arrested within the "G1" phase until the block to differentiation is removed - by the lifting of light limitation in the case of the R. vannielii swarmer cell.

Once the swarmer cell has differentiated to produce a stalked cell, this new reproductive cell faces a choice of whether to synthesise a swarmer cell or a second stalked cell. There is some evidence that this choice may be influenced by the carbon-dioxide tension in the medium with high values producing constitutive swarmer cell formation (Dow and France, 1980).

The study of the I period and control of differentiation is facilitated in R. vannielii compared to organisms such as E. coli by the ease in which large numbers of homogeneous swarmer cells can be obtained by selective filtration. Coulter Counter analysis shows that these swarmer cells differentiate synchronously (section 3.3). It is possible that in the case of bacteria such as E. coli which divide by binary fission into two apparently identical cells, that one of the cells is immature compared to the other and requires a period of maturation before initiation of DNA synthesis. The formation of an immature precursor cell has advantages as a dispersal phase and in being able to adapt to whatever environment it finds itself in before being committed

to cell division. This feature of the cell cycle is however difficult to study in E. coli because of the morphological near identity of the siblings produced at division whereas with R. vannielii the isolation of homogeneous swarmer cells is easy.

The existence of protein turnover in the light limited inhibited swarmer cell (section 3.6) and the relatively short life of this cell when inhibited is in keeping with a role for it in dispersal and adaptation to new environments rather than long term survival (section 3.7).

If the cell cycle were controlled by a developmental programme of sequential gene expression then the pattern of protein synthesis should change in a characteristic fashion through the cycle. The pattern of synthesis of 750 proteins through the cell cycle of E. coli B/r showed that none were synthesised at different rates at different stages of the cycle (Lutkenhaus et al., 1979). This may however be a consequence of the heterogeneity due to the non-equivalent nature of the sibling cells produced at division with one being an immature precursor cell. During the cell cyle of Caulobacter crescentus (Sheffery and Newton, 1981; Melhausen and Agabian, 1981; Ohta et al., 1982), fruiting body formation of myxobacteria (Kaiser et al., 1979), and during the fruiting body formation in Dicytostelium discoideum (Blumberg and Lodish, 1980b), significant numbers of proteins are synthesised only at defined periods of the cell cycle or only in particular cell types. Protein synthesis is periodic during the differentiation of Rhodomicrobium vannielii swarmer cells with qualitative and quantitative changes during swarmer cell maturation, stalk synthesis, daughter cell synthesis and during the formation of multicellular arrays (sections 3.9 and 3.10). There are also differences in the protein make up of different cell types, such as

swarmer cells, multicellular arrays and cells in early exponential phase growing without light limitation (section 3.8).

Proteins are also synthesised in the swarmer cell when it is inhibited by light limitation and although most of these are also synthesised in differentiating swarmer cells, a few, most notably an 11.5 K protein, are specific to the inhibited state and may be involved in the maintenance of inhibition. Many more proteins are specific to growing cells and may be involved in the cell differentiation or could possibly be involved in photosynthesis (sections 3.11 and 3.12).

How is this periodicity of protein synthesis controlled? In section 1.3 the evidence for a large number of possible control mechanisms for gene expression is outlined. These mechanisms range from changes in gene dosage and DNA structure through transcriptional and translational controls to proteolysis and the control of supramolecular structure synthesis.

At the level of the DNA structure, it appears that prokaryotic chromosomes contain nucleosome like structures although only part of the DNA is arranged in this way (Pettijohn, 1982). The chromosome is segregated into distinct domains of supercoiling that can be maintained independently and it is known that negative supercoiling enhances transcription (Zubay, 1980). Topoisomerases such as w protein can relax negatively supercoiled DNA and produce differential gene expression (Smith, 1981). Isolation of nucleoids from Caulobacter crescentus produced fast sedimenting nucleoids from swarmer cells and slow sedimentary nucleoids from stalked cells and the difference in structure may result in differential transcription (Swoboda et al., 1982).

At the level of transcription differential gene expression may result from modification of the DNA dependent RNA polymerase by the

synthesis of new sigma factors as for example in Bacillus subtilis (Doi, 1977; Losick and Pero, 1981; Talkington and Pero, 1979). In Escherichia coli the nucleotides ppGpp and ppApp induce opposing effects on the structural conformation of the RNA polymerase molecule and may thus alter its transcriptional specificity (Travers et al., 1980a).

The control of transcription is classically seen as involving repressor or activator proteins in the operator-promoter concept in which polymerase binding is either inhibited or facilitated. Well known examples include a specific repressor protein for the lac operon and an activator protein for the ara operon in Escherichia coli (Zubay, 1980). Another example occurs in the stringent response when the nucleotide ppGpp binds to the RNA polymerase and alters its transcriptional specificity. This effect is made long-lived by the synthesis of two proteins which also bind to the polymerase and produce a similar change in specificity (Richter et al., 1979; Travers, 1980).

At the level of translation control can be mediated by the secondary structure of mRNA which may need to be altered by specific proteins to allow binding to ribosomes. Examples of this mechanism are known from both Caulobacter crescentus and Escherichia coli (Marvil et al., 1980). The primary sequence of mRNA species may also affect gene expression both through frequency of initiation and the choice of codons (Grosjean and Fiers, 1982). Again at the translational level some ribosomal proteins act as repressors and can inhibit the translation of the ribosomal proteins whose genes are in the same operon as the repressor (Brot et al., 1981). In addition RNA polymerase acts as a repressor of the translation of the mRNA for the beta and beta' subunits (Lang-Yang and Zubay, 1981) and this mechanism could be more widespread.

Post-transcriptional processing of mRNA has also been invoked to

explain gene regulation with a specific nuclease RNAase III in Escherichia coli able to process dsRNA loops with the loss of messenger activity (Gottesman et al., 1982; Robertson, 1982).

Once protein has been synthesised control of gene expression is still possible by proteolysis. Gene expression can be altered by selective proteolysis of repressor or activator proteins, modification of RNA polymerase subunits and modification of initiation factors or ribosomal subunits which could alter translational control of specific messages (Maurize and Switzer, 1980).

Which of these possible means of control is responsible for the sequential gene expression observed in Rhodomicrobium vannielii is at present unknown and the problem may be difficult to solve given the large number of mechanisms mentioned above plus other less probable examples referred to in section 1.3.

An 11.5 K protein was only synthesised in Rhodomicrobium vannielii swarmer cells under light limitation and was degraded when the light limitation was removed or if the culture was incubated in the presence of air (section 3.11). The degradation of this protein correlated positively with ATP levels in the cell (section 3.16) raising the possibility that it was degraded by an energy-dependent process in a similar manner to proteins thought to be cell division inhibitors in Escherichia coli which are degraded by the lon(capR) ATP hydrolysis-dependent protease (Mizusawa and Gottesman, 1983; Schoemaker et al., 1982). The 11.5 K protein appears to be a soluble protein and no function has yet been assigned to it but it may be involved in the inhibition of cell division in the light limited cell. Antibody to this protein is available and an attempt to clone the gene coding for it into E. coli is being made. Purification by affinity chromatography

using the antibody should be possible but mutants will probably have to be isolated before its function can be definitely elucidated.

In summary this thesis has been concerned with protein synthesis and its regulation during the cell cycle of Rhodomicrobium vannielii. Evidence for the presence of an inhibited swarmer cell which has a dispersal and adaptation function is presented. The existence of sequential gene expression during differentiation of the swarmer cell is established and possible mechanisms of control discussed. A protein specific to the inhibited swarmer cell was discovered and its function and means of degradation discussed.

4. APPENDIX

4.1 Mathematical Model of Swarmer and Stalked Cell Numbers in a Simplified Culture of Rhodomicrobium vannielii (applicable to any dimorphic cell cycle)

Let the number of stalked cells at any time = T cells.

Let the number of swarmer cells at any time = W cells.

On division a stalked cell produces a swarmer cell and a stalked cell whereupon the swarmer develops into a stalked cell. So the increase in the number of stalked cells is only by the development of swarmer cells and the number of swarmers depends on the balance between production by division and differentiation into stalked cells.

So: $dT/dt = bW$ (1) and $dW/dt = aT - bW$ (2)

where "a" and "b" are constants in any particular growth conditions and are in inverse time units, so that "a^{-1}" approximates to the stalked cell generation time and "b^{-1}" approximates to the swarmer maturation time (see Figure 1.20(c)).

Differentiating equations (1) and (2) with respect to time:

$d^2T/dt^2 = bdW/dt$ (3) $d^2W/dt^2 = adT/dt - bdW/dt$ (4)

Substituting from (1) and (2) in (3)

$d^2T/dt^2 + bdT/dt - abT = 0$ (5)

The most general solution to (5) is:

$T = A_1e^{m_1t} + A_2e^{m_2t}$ where "m_1" and "m_2" are the roots of the quadratic

$m^2 + bm - ab = 0$

$m_1 = (\sqrt{b^2 + 4ab} - b)/2$ and $m_2 = -(\sqrt{b^2 + 4ab} + b)/2$

From equation (1) $W = A_1 m_1 e^{m_1 t}/b + A_2 m_2 e^{m_2 t}/b$

When $t = 0$, $T = T_0$, $W = W_0$ the initial values.

Therefore $T_0 = A_1 + A_2$ and $W_0 = A_1 m_1/b + A_2 m_2/b$

From this it follows taht:

$A_1 = (T_0 m_2 - bW_0)/(m_2 - m_1)$ and $A_2 = (bW_0 - T_0 m_1)/(m_2 - m_1)$

Therefore $T = (T_0 m_2 - bW_0)e^{m_1 t}/(m_2 - m_1) + (bW_0 - T_0 m_1)e^{m_2 t}/(m_2 - m_1)$ (6)

and $W = m_1(T_0 m_2 - bW_0)e^{m_1 t}/b(m_2 - m_1) + m_2(bW_0 - T_0 m_1)e^{m_2 t}/b(m_2 - m_1)$ (7)

If the growth time "t" is large the second term in equations (7) and (8) tends to zero because m_2 is negative.

$T_\infty = (T_0 m_2 - bW_0)e^{m_1 t}/(m_2 - m_1)$

$W_\infty = m_1(T_0 m_2 - bW_0)e^{m_1 t}/b(m_2 - m_1)$

From this it follows that the steady state proportion of swarmer cells

$W_\infty/W_\infty + T_\infty = m_1/(m_1 + b)$

and at this is a function of the stalked cell generation time and the swarmer maturation time. Figure 4.1 shows the proportion of swarmer cells in a culture with different swarmer maturation times in terms of the stalked cell generation time. The longer the maturation time becomes, or in other words the more inhibited development is, the greater the proportion of swarmer cells in the culture.

The equations for the growth of a population of synchronised swarmer cells may be derived by putting $T_0 = 0$ in equations (6) and (7).

$T = bW^0(e^{m_2 t} - e^{m_1 t})/(m_2 - m_1)$ (8)

$W = M_2 W_0 e^{m_2 t}/(m_2 - m_1) - M_1 W_0 e^{m_1 t}/(m_2 - m_1)$ (9)

Figures 4.2, 4.3 and 4.4 shows the growth of cultures of synchronised swarmer cells when the swarmer maturation times are various proportions of the stalked cell generation time. In each case the swarmer proportion drops to a level characteristic of the maturation time and

Figure 4.1

Model of the steady state proportion of swarmer cells in a simplified culture as a function of the swarmer cell development time in terms of the stalk cell generation time (a^{-1}).

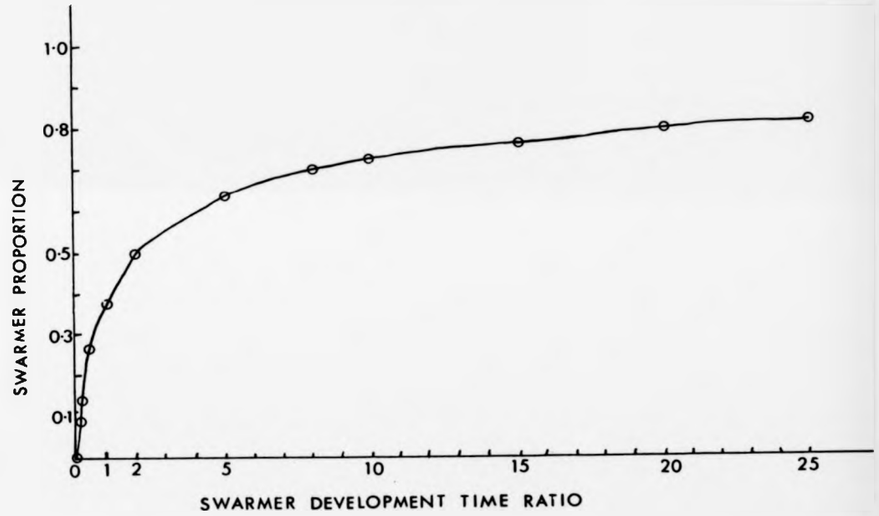


Figure 4.2

Model of growth of synchronised swarmer cells if the swarmer cell maturation time is equal to the stalked cell generation time.

Number of stalked cells (multiple of number of synchronised swarmers)

Number of swarmer cells (multiple of number of synchronised swarmers)

Proportionof swarmer cells.

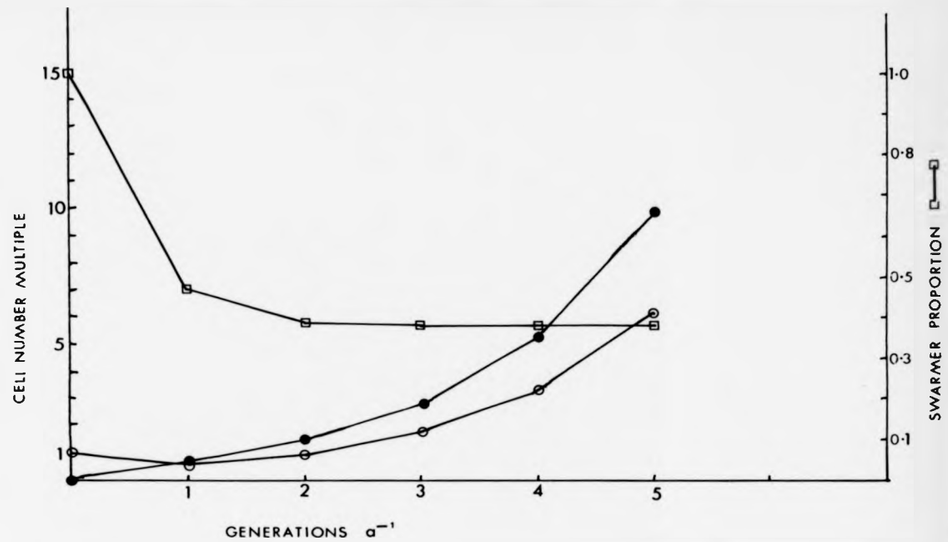
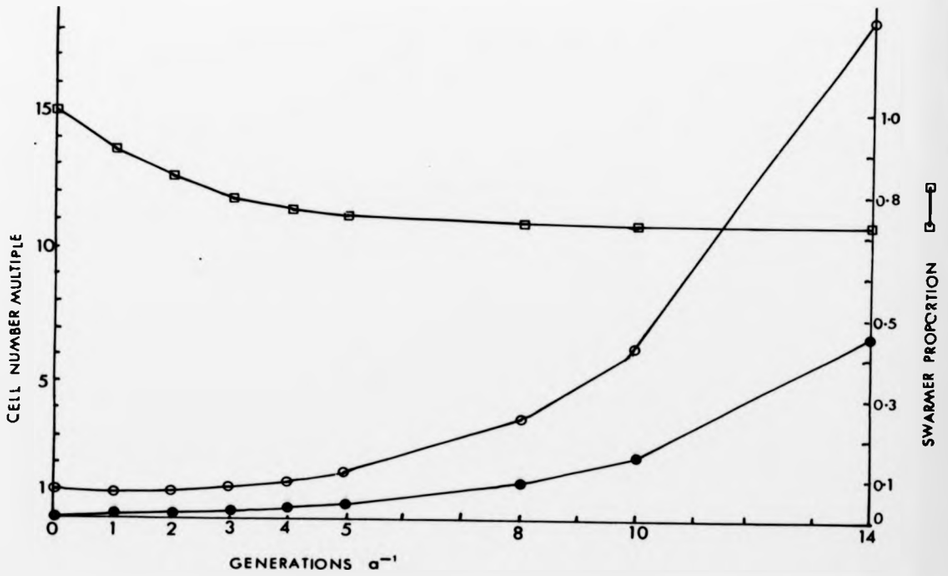
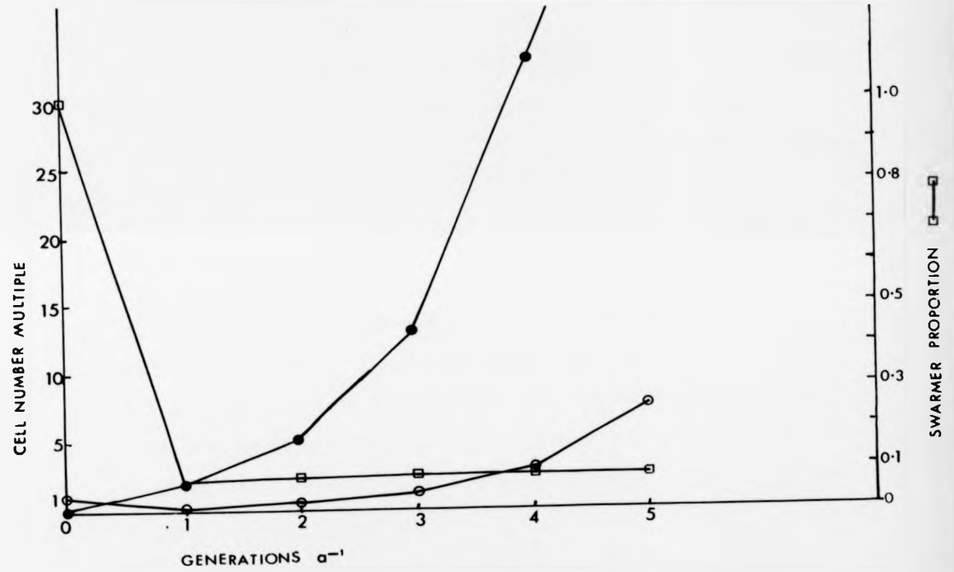


Figure 4.3

Model of growth of synchronised swarmer cells if the swarmer cell maturation time is 10 times the stalked cell generation time.




CELL NUMBER MULTIPLE
SWARMER PROPORTION
GENERATIONS a⁻¹
30
25
20
15
10
5
1
0
1
2
3
4
5
1·0
0·8
0·5
0·3
0·1
0

remains at that level as the numbers of swarmer cells and stalked cells increase. In the actual growth of a culture the proportion of swarmer cells increases again as the optical density of the culture increases due to the increase in cell number (see Figure 3.8). This can be modelled by increasing the maturation time as the cell number increases using an exponential function, so that:

Maturation time = Maturation time in dilute culture/EXP(T+W)/CONSTANT

where the constant is a parameter expressed in terms of cell number. In a similar way the stalked cell generation time is also increased with a different parameter in order that the culture enters stationary phase in the model. The differential equations resulting from this treatment cannot be integrated so the number of stalked and swarmer cells have to be calculated using a microcomputer.

4.2 Mathematical Model of Swarmer and Stalked Cell Numbers in a Complex Culture of Rhodomicrobium vannielii

In a complex culture a stalked cell has the choice of producing a swarmer cell or another stalked cell. So the increase in stalked cells is by development of swarmer cells and by direct production of stalked cells forming a multicellular array.

If on budding, a proportion "n" of stalked cells produce another stalked cell and a proportion "1-n" produce a swarmer cell then:

$dt/dt = bW + naT$ (1)

$dW/dt = (1-n)aT - bw$ (2)

where "a", "b", "W" and "T" are as defined in section 4.1.

Differentiating equations (1) and (2):

$d^2T/dt^2 = bdW/dt + nadT/dt$ (3)

$d^2W/dt^2 = (1-n)a\ dT/dt - bdW/dt$ (4)

Substituting from (1) and (2) into (3)

$d^2T/dt^2 + (b - an)\ dT/dt - abT = 0$ (5)

The most general solution to (5) is:

$T = A_1e^{m_1t} + A_2e^{m_2t}$ where "m_1" and "m_2" are the roots of the quadratic

$m^2 + (b - an)\ m - ab = 0$

Therefore $m_1 = (\sqrt{(b-an)^2 + 4ab} - b + an)/2$

$m_2 = -(\sqrt{(b-an)^2 + 4ab} + b - an)/2$

From (1) $W = (m_1-na)A_1e^{m_1t}/b + (m_2 - na)A_2e^{m_2t}/b$

When $t = 0$, $T = T_o$, $W = W_o$ as defined in section 4.1.

Therefore $T_o = A_1 + A_2$ and $W_o = A_1(m_1-na)/b + A_2(m_2-na)/b$

From this it follows that:

$A_1 = (T_o(m_2-na) - bW_o)/(m_2-m_1)$

$A_2 = (bW_o - T_o(m_1-na)/(m_2-m_1)$

Therefore $T = (T_o(m_2-na) - bW_o)e^{m_1t}/(m_2-m_1) + (bW_o - T_o(m_1-na))e^{m_2t}/(m_2-m_1)$

and $W = (m_1-na)(T_o(m_2-na) - bW_o)e^{m_1t}/b(m_2-m_1) + (m_2-na)(bW_o - T_o(m_1-na))e^{m_2t}/b(m_2-m_1)$

The growth equation for the simplified cycle is obtainable from this more general equation by putting $n = 0$.

The variables in the growth of a complex culture are the swarmer cell maturation time, the stalked cell generation time which are increased as the culture grows as described in section 4.1 and the proportion of stalk cells produced. This last variable can also be modulated in a similar way if required so that the proportion of swarmer cells produced increases as the optical density increases. A computer model was based on these variables to model the growth of a heterogeneous culture and

calculate the numbers and proportions of stalked and swarmer cells. The program is shown in Figure 4.13 and the following six parameters are needed as inputs.

(i) Swarmer cell muaturation time.

(ii) Stalked cell generation time.

(i) and (ii) are minimum times in a culture not inhibited in any way.

(iii) Proportion of stalked cells produced in a culture that is not inhibited.

(iv) Retardation of swarmer cell development parameter.

(v) Retardation of stalked cell development parameter

(vi) Variation of stalked/swarmer cell production parameter.

(iv), (v) and (vi) are expressed in cells ml^{-1} and control the rate at which increasing cell number alters variables (i), (ii) and (iii). When this model was run on a BBC Microcomputer it was found that by selection of the parameter (i) to (vi) a close match could be obtained with the experimental growth characteristics discussed in section 3.4. Obviously with six variable parameters there are many possible permutations but approximate observed values of 120 minutes for the swarmer maturation time and 360 minutes for the stalked cell generation time were used. For the retardation of stalked cell development parameter a value of 3.5×10^8 cells ml^{-1} was found to give the required stationary phase number of stalked cells and a value of 1×10^8 cells ml^{-1} for the retardation of swarmer cell development parameter gave the required number of swarmer cells (see Figure 4.5). This means that retardation of the differentiation concerned starts to take effect at about the level of these parameters. The proportion of stalked and swarmer cells produced initially is less critical and values from 0.9 to 0.98 give reasonable agreement. The parameter that increases the ratio

of swarmer cells to stalked cells produced as the culture grows gives best agreement at a value of 2×10^{10} cells ml^{-1} which is an order of magnitude higher than the stationary phase cell number reached so its effect is marginal. Lower values of this parameter such as 5×10^{9} (see Figure 4.10) and 1×10^{9} (see Figure 4.11) increase the number of swarmers in the stationary phase culture to levels characteristic of a simplified culture. Higher values such as 1×10^{11} (see Figure 4.12) depresses the number of swarmers somewhat but the increase still takes place showing that this is due to inhibition of swarmer development or maturation rather than an increase in the number of swarmers produced.

Explanation of Figures 4.5 to 4.12

Figures 4.5, 4.6 and 4.7 show the effect of increasing the stalked cell development retardation parameter on the number of stalked cells in the stationary phase.

Figures 4.8 and 4.9 show the effect of reducing the proportion of stalked cells produced on the number of swarmer cells in stationary phase.

Figures 4.10, 4.11 and 4.12 show the effect of varying the stalked/swarmer cell production parameter on the number of swarmer cells in stationary phase.

Figure 4.5

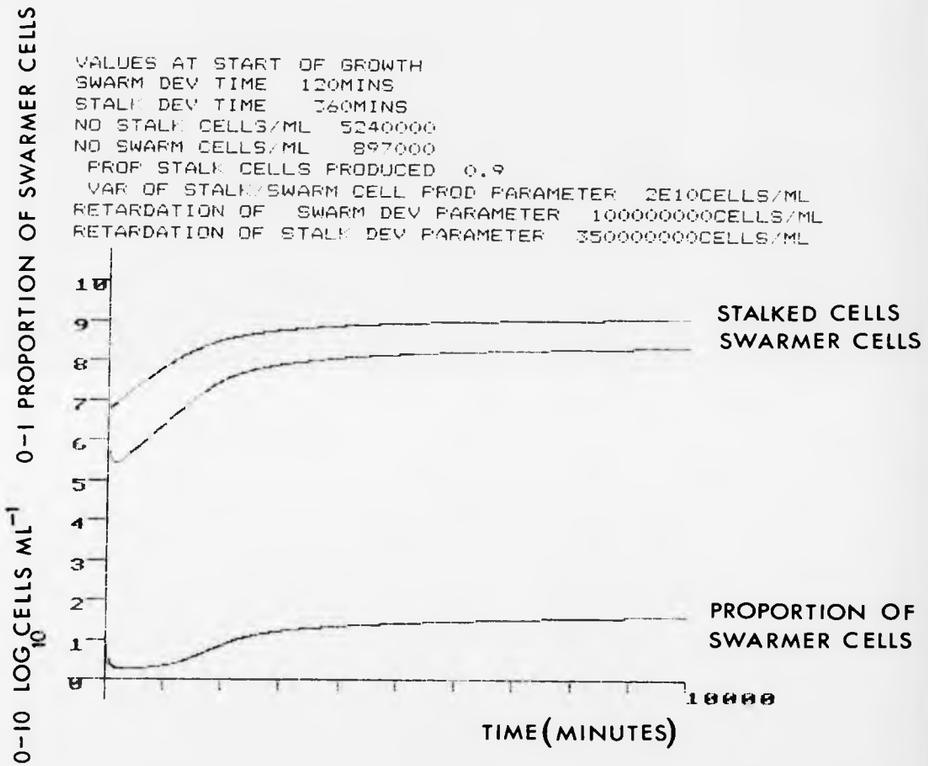


Figure 4.6

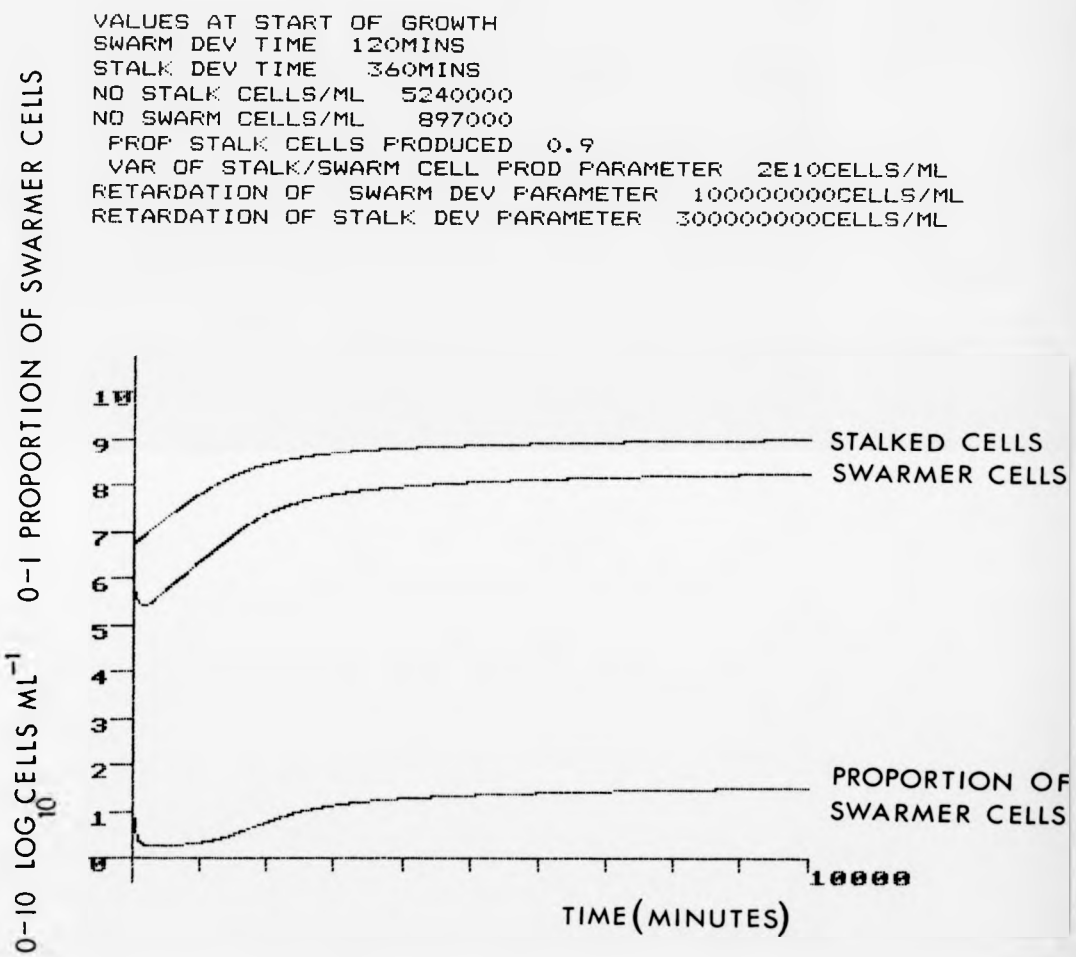

VALUES AT START OF GROWTH
SWARM DEV TIME 120MINS
STALK DEV TIME 360MINS
NO STALK CELLS/ML 5240000
NO SWARM CELLS/ML 897000
PROP STALK CELLS PRODUCED 0.9
VAR OF STALK/SWARM CELL PROD PARAMETER 2E10CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML
0-10 LOG10 CELLS ML-1
0-1 PROPORTION OF SWARMER CELLS
10
9
8
7
6
5
4
3
2
1
0
STALKED CELLS
SWARMER CELLS
PROPORTION OF SWARMER CELLS
10000
TIME (MINUTES)
VALUES AT END OF GROWTH
SWARM DEV TIME INFINITE
STALK DEV TIME 23882MINS
NO STALK CELLS/ML 1.0650247E9
NO SWARM CELLS/ML 193410516
PROP STALK CELLS PRODUCED 0.845115245
VAR OF STALK/SWARM CELL PROD PARAMETER 2E10CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML

Figure 4.7

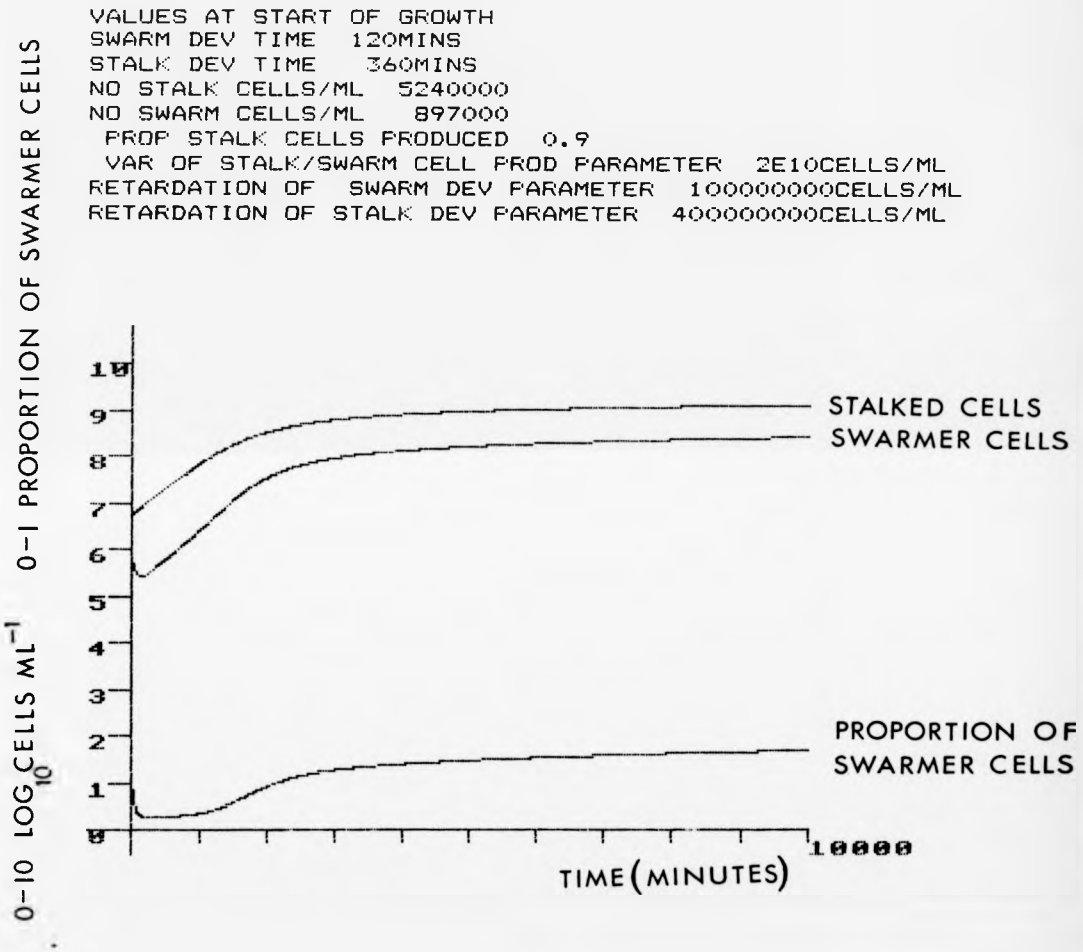

VALUES AT START OF GROWTH
SWARM DEV TIME 120MINS
STALK DEV TIME 360MINS
NO STALK CELLS/ML 5240000
NO SWARM CELLS/ML 897000
PROP STALK CELLS PRODUCED 0.9
VAR OF STALK/SWARM CELL PROD PARAMETER 2E10CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 400000000CELLS/ML
0-10 LOG10 CELLS ML-1
0-1 PROPORTION OF SWARMER CELLS
10
9
8
7
6
5
4
3
2
1
0
STALKED CELLS
SWARMER CELLS
PROPORTION OF SWARMER CELLS
10000
TIME (MINUTES)
VALUES AT END OF GROWTH
SWARM DEV TIME INFINITE
STALK DEV TIME 23131MINS
NO STALK CELLS/ML 1.38123975E9
NO SWARM CELLS/ML 283892268
PROP STALK CELLS PRODUCED 0.828103513
VAR OF STALK/SWARM CELL PROD PARAMETER 2E10CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 400000000CELLS/ML

Figure 4.8

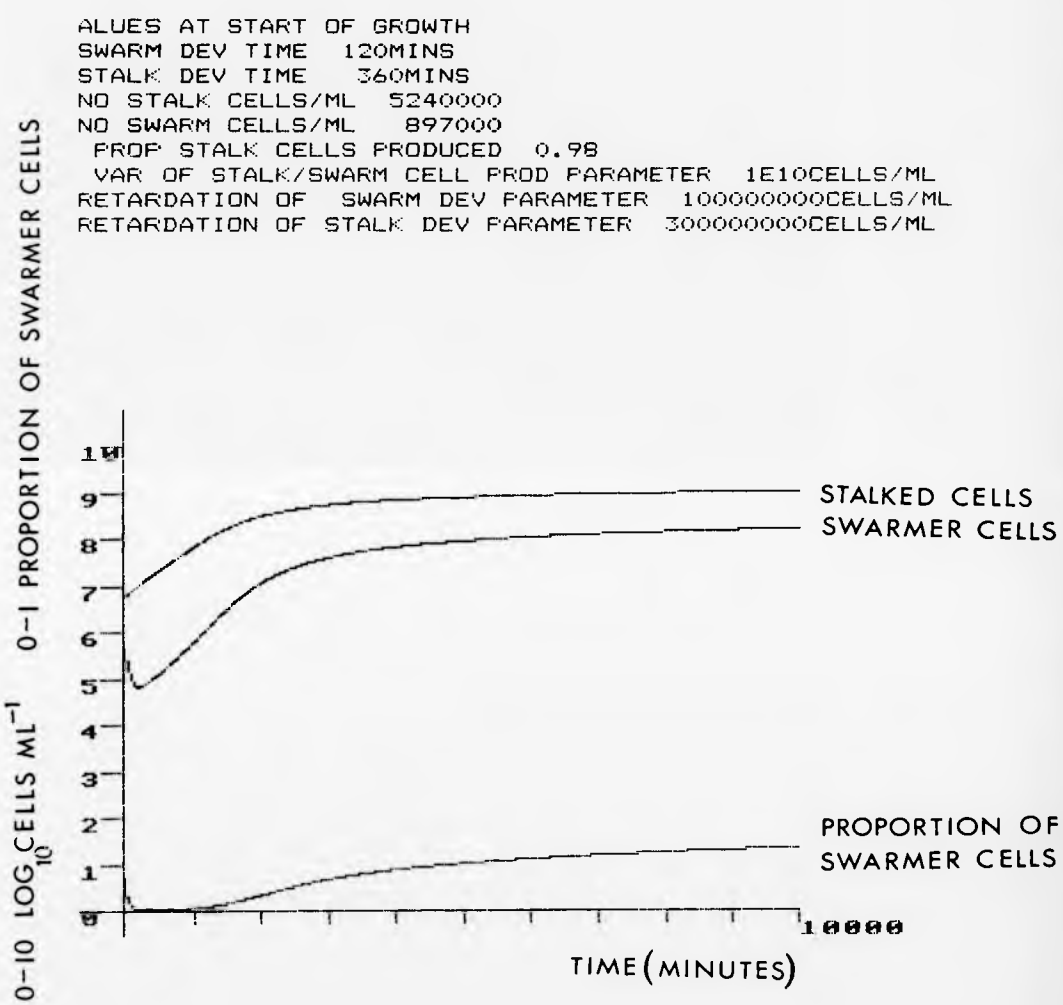
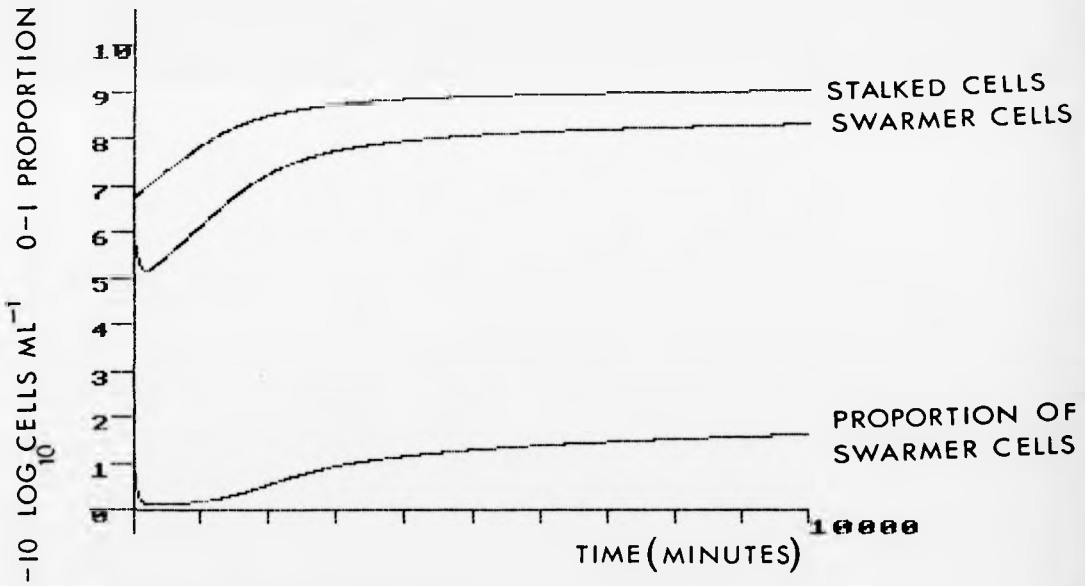

ALUES AT START OF GROWTH
SWARM DEV TIME 120MINS
STALK DEV TIME 360MINS
NO STALK CELLS/ML 5240000
NO SWARM CELLS/ML 897000
PROP STALK CELLS PRODUCED 0.98
VAR OF STALK/SWARM CELL PROD PARAMETER 1E10CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML
0-10 LOG10 CELLS ML-1
0-1 PROPORTION OF SWARMER CELLS
STALKED CELLS
SWARMER CELLS
PROPORTION OF SWARMER CELLS
10000
TIME (MINUTES)
VALUES AT END OF GROWTH
SWARM DEV TIME INFINITE
STALK DEV TIME 26668MINS
NO STALK CELLS/ML 1.11547474E9
NO SWARM CELLS/ML 176055596
PROP STALK CELLS PRODUCED 0.861262674
VAR OF STALK/SWARM CELL PROD PARAMETER 1E10CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML

Figure 4.9

VALUES AT START OF GROWTH
SWARM DEV TIME 120MINS
STALK DEV TIME 360MINS
NO STALK CELLS/ML 5240000
NO SWARM CELLS/ML 897000
PROP STALK CELLS PRODUCED 0.95
VAR OF STALK/SWARM CELL PROD PARAMETER 1E10CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML



VALUES AT END OF GROWTH
SWARM DEV TIME INFINITE
STALK DEV TIME 25708MINS
NO STALK CELLS/ML 1.0733232E9
NO SWARM CELLS/ML 207218019
PROP STALK CELLS PRODUCED 0.835815473
VAR OF STALK/SWARM CELL PROD PARAMETER 1E10CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML

Figure 4.10

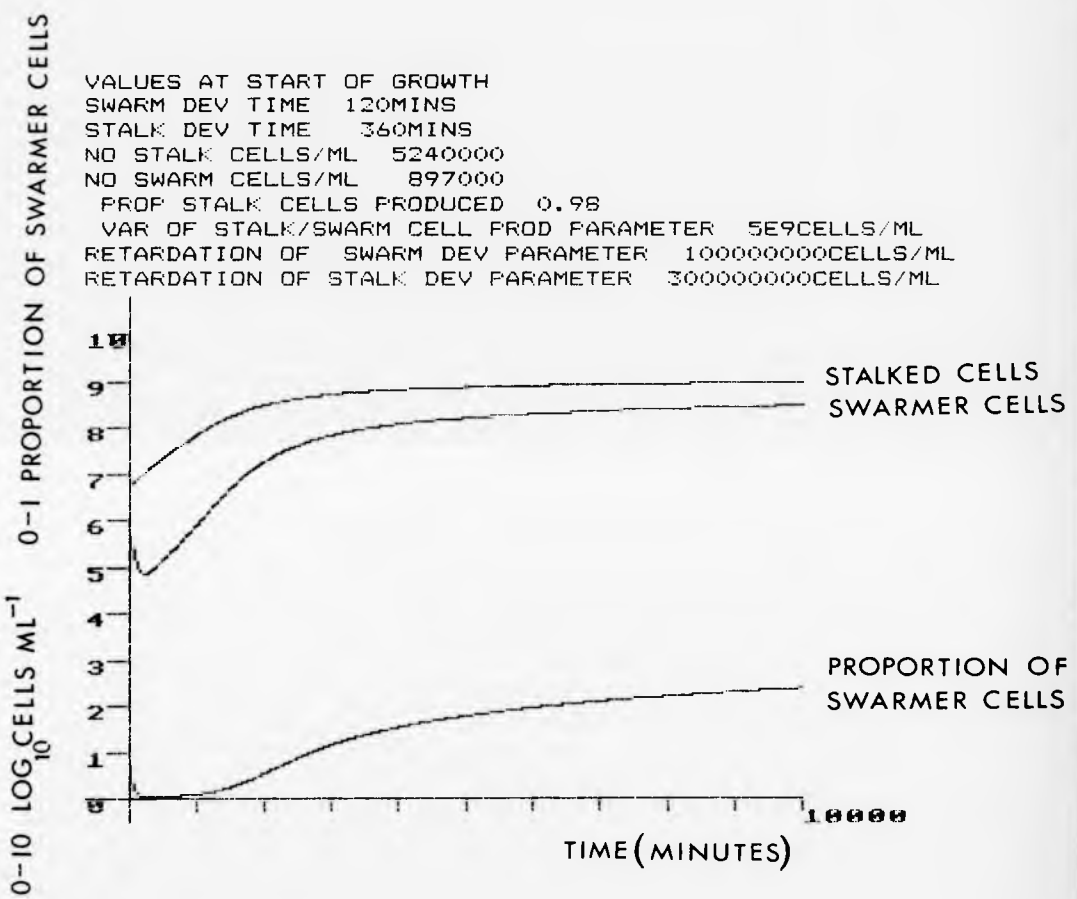

VALUES AT START OF GROWTH
SWARM DEV TIME 120MINS
STALK DEV TIME 360MINS
NO STALK CELLS/ML 5240000
NO SWARM CELLS/ML 897000
PROP STALK CELLS PRODUCED 0.98
VAR OF STALK/SWARM CELL PROD PARAMETER 5E9CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML
0-10 LOG10 CELLS ML-1 0-1 PROPORTION OF SWARMER CELLS
STALKED CELLS
SWARMER CELLS
PROPORTION OF SWARMER CELLS
TIME (MINUTES)
VALUES AT END OF GROWTH
SWARM DEV TIME INFINITE
STALK DEV TIME 27656MINS
NO STALK CELLS/ML 989790630
NO SWARM CELLS/ML 312659997
PROP STALK CELLS PRODUCED 0.755260291
VAR OF STALK/SWARM CELL PROD PARAMETER 5E9CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML

Figure 4.11

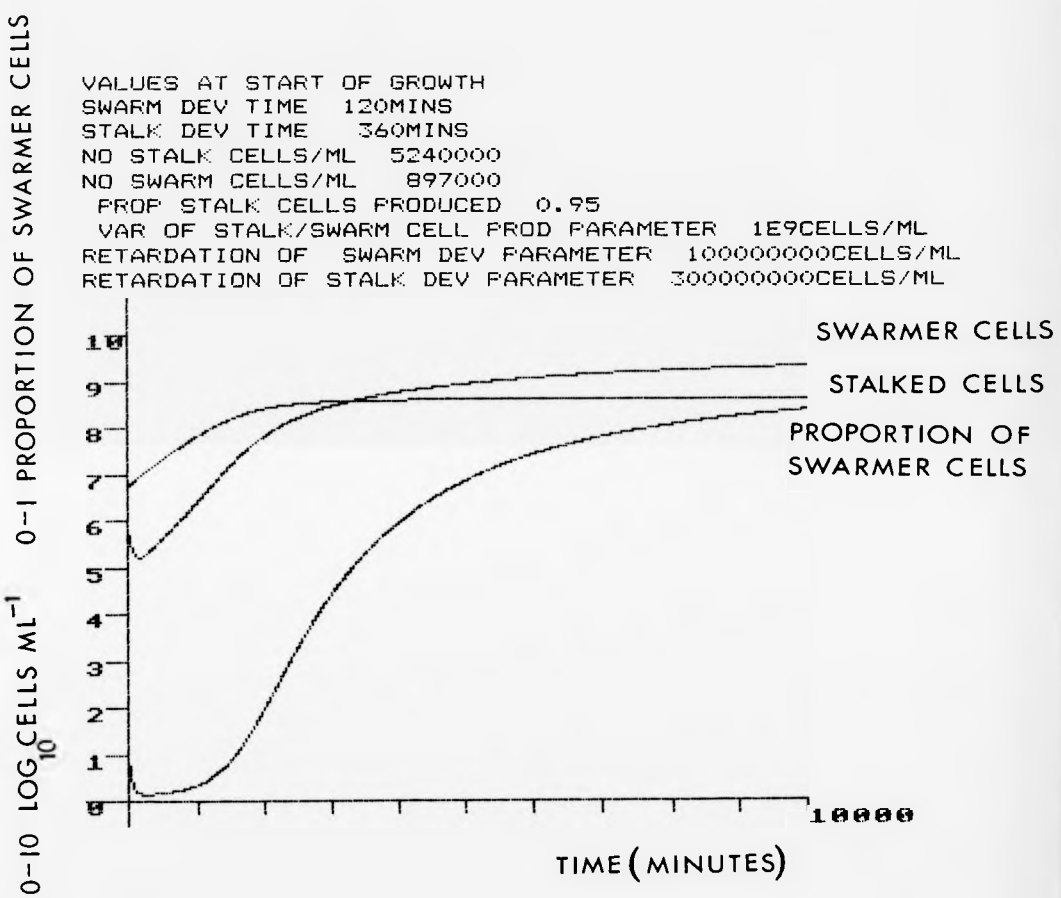
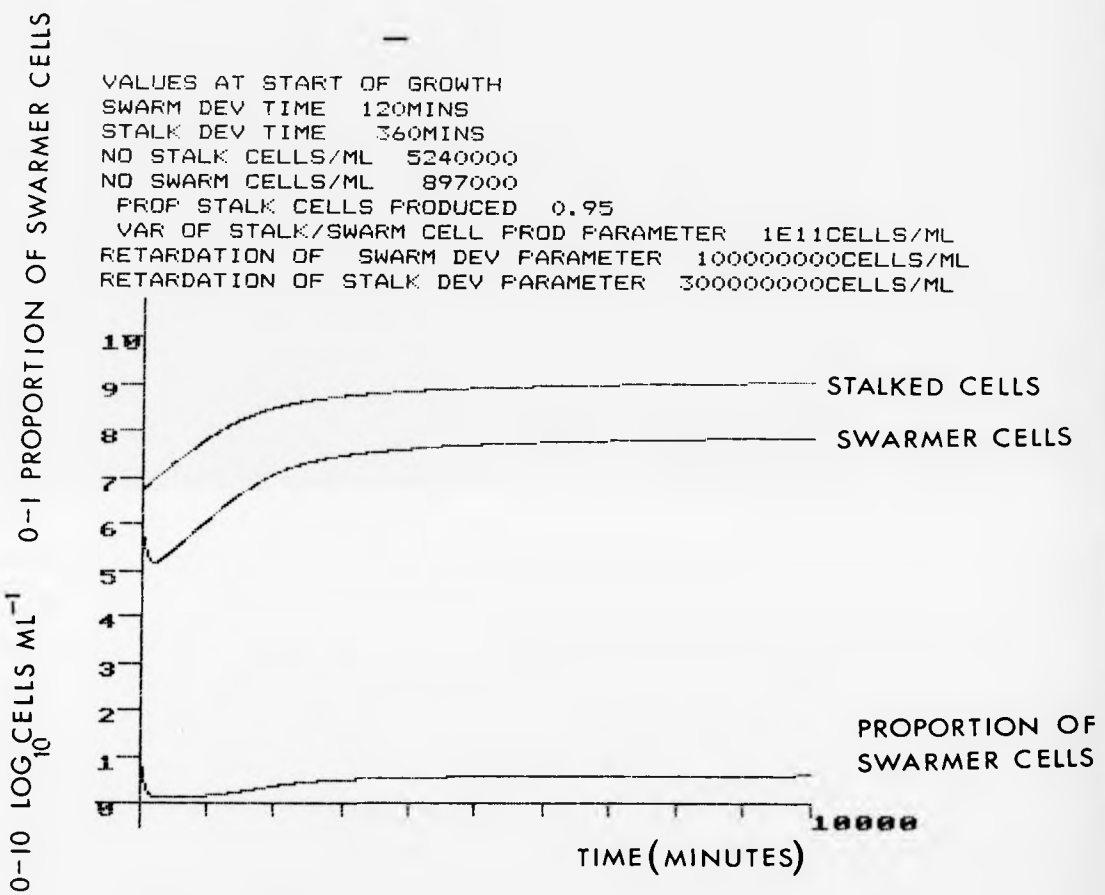

VALUES AT START OF GROWTH
SWARM DEV TIME 120MINS
STALK DEV TIME 360MINS
NO STALK CELLS/ML 5240000
NO SWARM CELLS/ML 897000
PROP STALK CELLS PRODUCED 0.95
VAR OF STALK/SWARM CELL PROD PARAMETER 1E9CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML
SWARMER CELLS
STALKED CELLS
PROPORTION OF SWARMER CELLS
0-10 LOG10 CELLS ML-1
0-1 PROPORTION OF SWARMER CELLS
10
9
8
7
6
5
4
3
2
1
0
10000
TIME (MINUTES)
VALUES AT END OF GROWTH
SWARM DEV TIME INFINITE
STALK DEV TIME INFINITE
NO STALK CELLS/ML 421921162
NO SWARM CELLS/ML 2.2158571E9
PROP STALK CELLS PRODUCED 6.79439924E-2
VAR OF STALK/SWARM CELL PROD PARAMETER 1E9CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML

Figure 4.12



VALUES AT END OF GROWTH
SWARM DEV TIME INFINITE
STALK DEV TIME 25279MINS
NO STALK CELLS/ML 1.19668862E9
NO SWARM CELLS/ML 78794950.8
PROP STALK CELLS PRODUCED 0.937959854
VAR OF STALK/SWARM CELL PROD PARAMETER 1E11CELLS/ML
RETARDATION OF SWARM DEV PARAMETER 100000000CELLS/ML
RETARDATION OF STALK DEV PARAMETER 300000000CELLS/ML

Figure 4.13

Computer program to calculate swarmer and stalked cell numbers.

```
 1CLS
 2*LOAD"DUMP"
 5REM THIS PROGRAM TO STATIONARY PHASE
 10REM MODEL OF SWARMER AND STALK CELL NUMBERS IN BUDDING BACTERIA
 20 PRINT"SWARM CELL DEVELOPMENT TIME IN MINUTES"
 30INPUT C
 40 B=10/C
 45 E=10/C
 50PRINT"STALK CELL REPRODUCTION      TIME IN MINUTES"
 60INPUT D
 70 A=10/D
 75 F=10/D
 80PRINT"STARTING  NUMBER OF SWARM    CELLS PER ML"
 90INPUT W
 100 PRINT"STARTING NUMBER OF STALK    CELLS PER ML"
 105  I%=0
 110INPUT T
 120REM ON DIVISION STALK CELL CAN     PRODUCE A STALK CELL OR SWARM CELL
 130 PRINT"PROPORTION OF STALK  CELLS  PRODUCING A STALK CELL-ZERO TO ONE"
 140 INPUT P
 145 N=P
 150REM FOR SIMPLE CELL CYCLE OR       CAULOBACTER ETC N IS ZERO
 152 PRINT"VARIATION OF STALK/SWARM CELLPRODUCTION PARAMETER CELLS/ML"
 155 INPUT S
 160 PRINT"RETARDATION OF SWARM CELL   DEVELOPMENT PARAMETER-CELLS PER ML"
 170INPUT K
 175 PRINT "RETARDATION OF STALK CELL    DEVELOPMENT PARAMETER-CELLS PER ML"
 177 INPUT J
 180REM SWARM CELL DEVELOPMENT IS      RETARDED BY CELLS PER ML
 190 PRINT"HOW LONG DO YOU WISH THE    CULTURE TO GROW-MINUTES"
 200INPUT L
 205 VDU2:PRINT"VALUES AT START OF GROWTH":VDU3
 210 PROCPRINT
 215MODE 1:PROCSCALES
 240 FOR I=1 TO L/10
 250  M1=(SQR((B-A*N)^2+4*A*B)-B+A*N)/2
 260 M2=-(SQR((B-A*N)^2+4*A*N)+B-A*N)/2
 270T=(((T*(M2-N*A)-B*W)*EXP(M1))/(M2-M1))+(((B*W-T*(M1-N*A))*EXP(M2))/(M2-M1))
 280W=(((M1-N*A)*(T*(M2-N*A)-B*W)*EXP(M1))/(B*(M2-M1)))+(((M2-N*A)*(B*W-T*(M1-N
*A))*EXP(M2))/(B*(M2-M1)))
 290B=E/EXP((T+W)/K)
 295 N=P/EXP((T+W)/S)
 300 A=F/EXP((T+W)/J)
 310PROCDRAW
 320NEXT I:VDU2:CALL&A00:PRINT"VALUES AT END OF GROWTH":VDU3:PROCPRINT
 325 END
 330 DEFPROCDRAW
 340X%=I+64:Y1%=INT(LOG(T)*90+.5)+48
 350Y2%=INT(LOG(W)*90+0.5)+48
 360 Y3%=INT((W/(W+T))*900+.5)+48
 370 GCOL0,1:IF X%=65 MOVE X%,Y1%:GOTO400
 380MOVE X%-1,Y1A%
 390DRAW X%,Y1%
 400Y1A%=Y1%
 410 GCOL0,2:IF X%=65 MOVE X%,Y2%:GOTO440
 420MOVE X%-1,Y2A%
 430DRAW X%,Y2%
 440Y2A%=Y2%
 450 GCOL0,3:IF X%=65 MOVE X%,Y3%:GOTO500
 480MOVE X%-1,Y3A%
 490DRAW X%,Y3%
 500Y3A%=Y3%
 510ENDPROC
 600 DEF PROCSCALES:N%=0
 605VDU5
 610 MOVE0,48:DRAW(L/10)+64,48:REM X AXIS
 620MOVE64,0:DRAW64,1024:REM Y AXIS
 630FOR I%=48 TO 948 STEP 90
 640MOVE64,I%:DRAW32,I%:MOVE0,I%:PRINT;N%
 645N%=N%+1
 650NEXT
 660FOR I%= 64 TO (L/10)+64 STEP L/100
 670MOVEI%,48:DRAWI%,24:NEXT:PRINT;L:VDU4
 680ENDPROC
 700 DEF PROCPRINT
 710 VDU2
 720 IF (10/B)<100000 THEN PRINT"SWARM DEV TIME  ";INT((10/B)+.5)"MINS" ELSE PR
INT"SWARM DEV TIME  INFINITE "
 730 IF (10/A)<100000 THEN PRINT"STALK DEV TIME   ";INT((10/A)+.5)"MINS" ELSE P
RINT "STALK DEV TIME INFINITE "
 740 PRINT"NO STALK CELLS/ML  ";T
 750  PRINT"NO SWARM CELLS/ML   ";W
 755PRINT" PROP STALK CELLS PRODUCED  ";N
 760 PRINT" VAR OF STALK/SWARM CELL PROD PARAMETER  ";S "CELLS/ML"
 765PRINT "RETARDATION OF  SWARM DEV PARAMETER  ";K"CELLS/ML"
 770PRINT"RETARDATION OF STALK DEV PARAMETER  ";J "CELLS/ML"
 780VDU3
 790 ENDPROC
```

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JOURNAL TITLE ABBREVIATIONS

Adv. Microbial. Phys.	Advances in Microbial Physiology
Anal. Biochem.	Analytical Biochemistry
Ann. Rev. Genetics	Annual Review of Genetics
Ann. Rev. Microbiol.	Annual Review of Microbiology
Arch. Biochem. Biophys.	Archives of Biochemistry and Biophysics
Arch. Microbiol.	Archives of Microbiology
Bact. Rev.	Bacterial Reviews
B.B.A.	Biochemica et Biophysica Acta
B.B.R.C.	Biochemical and Biophysical Research Communications
Curr. Top. Microbiol. Immunol.	Current Topics in Microbiology and Immunology
Dev. Biol.	Developmental Biology
Eur. J. Biochem.	European Journal of Biochemistry
F.E.B.S. Letters	Federation of European Biochemical Societies Letters
F.E.M.S. Microbiology Letters	Federation of European Microbiological Societies Microbiology Letters
J. Bact.	Journal of Bacteriology
J. Biol. Chem.	Journal of Biological Chemistry
J. Cell. Sci.	Journal of Cell Science
J. Gen. Appl. Microbiol.	Journal of General and Applied Microbiology
J.G.M.	Journal of General Microbiology
J.M.B.	Journal of Molecular Biology
M.G.G.	Molecular and General Genetics
P.N.A.S.	Proceedings of the National Academy of Sciences of the United States of America
T.I.B.S.	Trends in Biochemical Sciences