MORPHOGENESIS AND DIFFERENTIATION

IN

RHODOMICROBIUM

by

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SUMMARY

Differentiation and morphogenesis in the budding photosynthetic bacterium. Rhodomicrobium vannielii (Rm5) was investigated, using batch and continuous culture techniques. A previously undocumented 'simplified' vegetative cell cycle was identified. 'Simplified' Rm 5 cells, with a developmental cycle resembling that of the prosthecate Hyphomicrobium, were isolated from the late exponential growth phase of batch cultures, or from continuous cultures grown under high CO_2 tensions. Expression of this cell type appears to be under environmental control, although the possibility that such cells may be mutants has not been overlooked. Previous studies have associated swarm cell production with mother cells comprising multicellular arrays, but the existence of 'simplified' cells suggests that microcolony formation is not a prerequisite for swarm cell synthesis. The characteristics of this unusual cell type and the morphology and physiology were compared with cells of Rhodomicrobium expressing the 'normal' developmental cycle. Soluble protein extracts of 'simplified' Rm 5 cells were compared with those of the other cell types by polyacrylamide gel electrophoresis.

Since reproducibility of results was difficult to achieve under batch culture conditions, the possibility of using a chemostat to perform simple physiological studies and optimise growth conditions was investigated. Experiments were performed in order to try and elucidate whether or not 'simplified' Rm 5 cells were mutations, or a form of cellular expression in response to particular environmental conditions. Although growth of Rm 5 is accompanied by obligate differentiation, life cycle variations such as exospore production and swarm cell formation are almost certainly environmentally induced. Preliminary investigations were made as to the effect of different environmental conditions on cellular expression in Rm 5. Light intensity, pH and CO_2 tension were involved with swarm cell production and development, whereas the presence of trace elements was a probable requirement for successful exospore formation.

ACKNOWLEDGEMENTS

CONTENTS

DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself under the supervision of Professor R. Whittenbury and Dr. C.S. Dow, and all sources of information have been specifically acknowledged by means of references.

Andrew D. France

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Section 1.   INTRODUCTION

(i)   General introduction
(ii) Model systems of increasing complexity
      Escherichia coli
      Rhodopseudomonas acidophila
      Rhodopseudomonas palustris
      Rhodomicrobium vannielii
(iii) Environmental control of differentiation
(iv) Regulation of differentiation at the molecular level
(v)   Cyclic nucleotides and their involvement in differentiation
(vi) Rhodomicrobium Rm 5 .   A useful model for studying
      morphogenesis and differentiation

(i) General introduction

Cellular morphogenesis, differentiation and development have been widely studied in both procaryotes (Donachie et al., 1973; Shapiro et al., 1971; Kimberlin-Hariri et al., 1977) and eucaryotes (Mitchison, 1969; Garrod and Ashworth, 1969; Gurdon and Woodland, 1968; Gurdon, 1974). Living cells, whether they function as unicellular organisms or are components of complex tissues and organs are not static units, but can change morphologically, adapt to environmental conditions by biochemical alteration, or express different functions at different times in the life cycle. The biochemistry of differentiation and its control in higher organisms has proved particularly difficult to study, largely as a result of the fact that many of the biological aspects of such organisms, particularly at the cellular level, are still poorly understood. A reasonable understanding of these processes is of paramount importance to the numerous facets of medical physiology (i.e. spermatogenesis, oogenesis, embryology and tumour tissue growth to name but a few). Unravelling the complex mechanisms involved in the development of a human being and their cellular control is obviously the ultimate goal. Homo sapiens, however, make a rather poor model system and the organism does not lend itself easily to experimentation ! In general the 'lower' the organism the more is known about its molecular biology. It might be argued, therefore, that a fuller understanding of the regulatory mechanisms of differentiating procaryotic cells may provide pertinent information about the principles, if not the processes of morphogenesis and differentiation in eucaryotes. Bonner (1973), however, considers studies of differentiation in 'lower' organisms to be valid only towards a greater understanding of the processes in these organisms, and questions the relevance of such extrapolations, arguing that the difference between procaryotic and eucaryotic cells reflects a basic discontinuity in cellular evolution. Although relatively few bacteria carry out a well defined morphogenesis other than cell division during their normal life cycles, such differentiation patterns can be categorized according to their inducible or obligatory nature. Such changes may be under environmental

control as in some bacteria, e.g. endospore formation in Bacillus
(Sudo and Dworkin, 1973), whereas in others morphogenetic events
are an obligate part of the cell cycle and occur in a fixed temporal
sequence independent of growth conditions, e.g. morphological alter-
ations in Caulobacter such as stalk, pili and flagella formation
(Shapiro, 1976).

(ii) Model systems of increasing complexity

To be a useful model system for cell development, a bacterium must
obey the following criteria:-

(a) Possess a simple and well defined life cycle

(b) Be easily synchronised

(c) Be able to grow on defined media so that biochemical events can be
     correlated with morphological development

(d) Be amenable to genetic manipulation

Bacteria that fulfil these criteria include Escherichia coli, Bacillus
subtilis and Caulobacter crescentus. An increasing diversity of
differentiation is reveals in this series: Escherichia coli → Bacillus
subtilis → Rhodopseudomonas acidophila → Rhodopseudomonas palustris →
Rhodomicrobium vannielii, which is worthy of discussion and provides a
useful illustration of some of the differentiation patterns available.

E. coli, a rod shaped, Gram negative, non-photosynthetic, facultative
anaerobe, is perhaps the most widely studied of all bacteria. The
transition of an E. coli F^- cell to a piliated F^+ cell by the acquisition of
an F episome serves as a simple example of localised morphogenesis which
is independent of the cell cycle (Valentine et al., 1969), whereas flagella
formation, which appears to be sensitive to catabolite repression, is an
example of inducible morphogenesis (Dobrogosz and Hamilton, 1971;
Yokoto and Gots, 1970). The morphogenetic capacity of this organism
has been studied by converting the rod shaped cells into spheres (L. forms)
by the use of antibiotics, and observing whether the cells possess the
ability for sphere-rod morphogenesis (Goodell and Schwartz, 1975).
L forms have been observed to reproduce by budding or multiple fission,
and reversion back to the normal form appears to be influenced by

environmental conditions (Smith, 1969). During the normal cycle, however, the only defined morphogenesis is cell division which proceeds by binary transverse fission (Gilleland and Murray, 1975).

Bacillus subtilis perhaps lends itself better to work on cellular differentiation than E. coli. This organism is a strictly aerobic, Gram positive chemoheterotrophic rod which divides by binary fission. However, under certain conditions it can present itself as a complex differentiating system (Hansen et al., 1970; Freese, 1972; Szulmajster, 1973; Dawes and Hansen, 1972). The development of an endospore occurs in a complex morphological sequence (Fig. 1.1) shortly after the cells enter the stationary phase of growth and begins with the appearance of a relatively transparent forespore, the formation of which is preceded by characteristic nuclear changes. The pair of nucleoids in the vegetative cell can be seen to fuse to give a rod shaped structure, which then divides, part of the DNA entering the developing spore and part remaining in the sporangium. Division of the rod shaped nucleus is accompanied by unequal cell division, and a new septum grows across the wall near one pole, segregating in the smaller cell a complete genome. The newly formed septum then grows back around the pole of the smaller cell, which becomes cut off from, and completely enclosed by, the larger cell. Once completely enclosed by the larger cell, the forespore develops rapidly into a mature endospore by the formation of several outer layers which include the cortex and spore coat. The cytological transformations are accompanied by alterations in the chemical and enzymatic composition of the cell. The spore coat has a different composition from the vegetative cell wall and contains calcium dipicolinate. Enzyme activities are also markedly different from those found in the vegetative cell. This change is believed to be brought about by the expression of new classes of genes during sporulation and the suppression of vegetative genes, and is discussed later. The different-iation of a vegetative cell into a spore is an example of reversible differentiation, the reversion of which is characterised by the temporal sequence of morphogenetic events occurring during germination. This
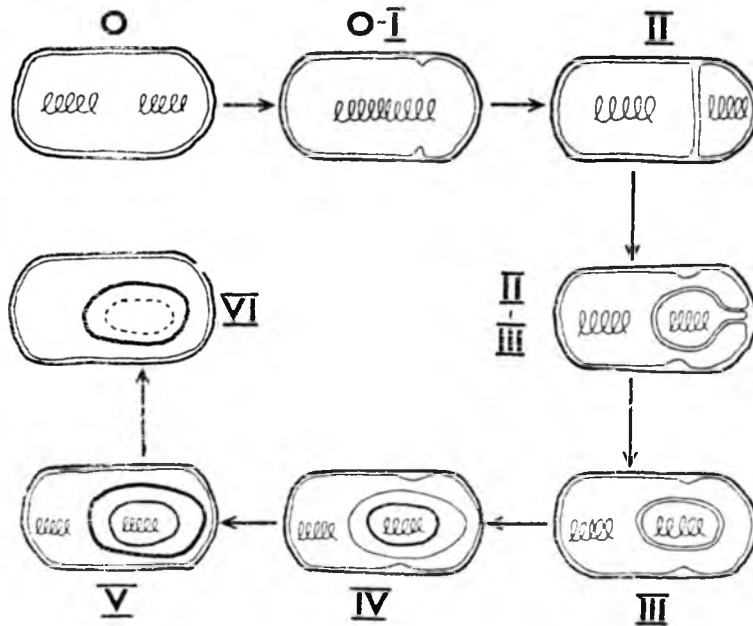
Fig. 1.1

Morphological changes associated with the stages of sporulation (after
Piggot and Coote, 1976).

0   Vegetative cell.   Pair of nucleoids.

I   Condensation of nucleoids.

II   Division of nucleoids.   Septum completion.

III   Formation of protoplast within mother cell.

IV   Primordial germ cell wall and cortex deposition.

V-VI   Spore maturation.

can be triggered, i.e.activated, by heat shock, mechanical treatment (Curran and Evans, 1945; Fitz-James, 1955; Levinson and Wrigley, 1960), and chemicals such as ethanol, urea-mercaptoethanol, as well as various acids and alkalis (Levinson and Hyatt, 1964, 1969; Keynan et al., 1964; Somerville et al., 1970). Subsequent germination is characterised by a series of unique structural and physiological changes. Both heat resistance and refractility of the spores is lost, but the ability to absorb stain is recovered. These alterations occur very rapidly and are accompanied by the disappearance of the cortex and the liberation of soluble organic materials including dipicolinic acid, proteins, peptides and mucopeptides. If the nutrients necessary for growth are present, germination is followed by the conversion of the spore back into a vegetative cell, and after about 30 min the outer spore coat ruptures and a vegetative cell emerges.

Although E. coli and B. subtilis are perhaps the best understood bacteria in terms of molecular biology, other relatively neglected bacteria with complex fine structures and morphologically distinctive cell cycles appear to have far greater potentials as models for the study of morpho-genesis and differentiation. Certain purple non-sulphur bacteria which reproduce by budding provide more diverse model systems. Such organisms have been widely reviewed (Conti and Hirsch, 1965; Schmidt, 1971; Staley,1974;Hirsch, 1972,1974Whittenbury and Dow, 1977). In essence all budding bacteria have adopted obligate asymmetric polar growth and do not differ fundamentally from other bacteria in respect of cell wall growth.

Bacteria, by definition, divide by fission, and this is preceded by distinct cell wall growth which increases the length or diameter of the cells. Bud formation prior to fission is a special case of wall growth and can be described as follows. The parental wall is locally weakened, allowing wall and membrane growth in that restricted region. Consequently, virtually all of the wall and membrane of a daughter cell is of newly synthesised material. The new cell is formed at a specific location on the surface of the mother cell, either directly from the pole of the mother cell as in Rhodopseudomonas acidophila or at the end of a tube which has first been formed by the mother cell as in Rhodopseudomonas palustris and Rhodomicrobium vannielii.
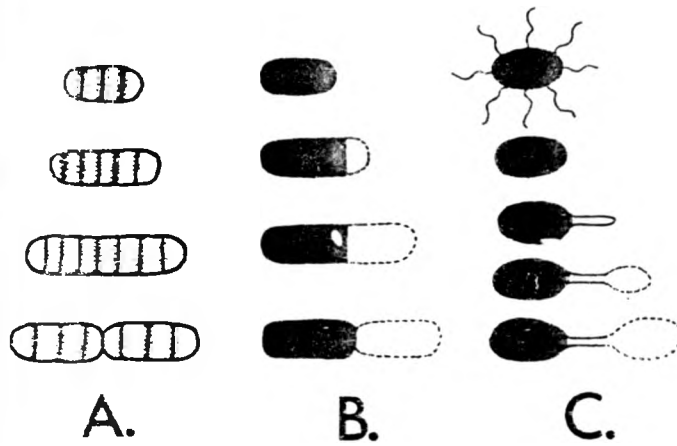
Fig. 1.2

Diagrammatic representation of cell wall growth processes.

(A)   Intercalatory growth of E. coli. Short generation time (less
        than 40 min).
(B)   Polar growth of E. coli. Long generation time (greater than
        60 min).
(C)   Obligate polar growth of the 'budding' bacterium
        Rhodomicrobium vannielii.

Each mother cell produces several buds, one after the other, and can be considered to undergo a process of aging since it can only give rise to a certain number of offspring (Whittenbury and Dow, 1977). This, however, is not the case with E. coli and other rod shaped bacteria which normally grow from multiple growth points along the envelope approximating to intercalatory growth (Beachey and Cole, 1966; Donachie and Begg, 1970; Autissier et al., 1971). Here the two progeny cells are of equal age and since one cannot be considered to be older than the other, i.e. there is an equal distribution of old and new material, such cells are therefore immortal. Nevertheless, polar growth of E. coli has been demonstrated (Donachie and Begg, 1970; Donachie et al., 1973). In minimal medium with a generation time of 60 minutes, E. coli will extend only from one pole. The only difference between budding bacteria and E. coli in this respect is that in budding bacteria division is usually asymmetric (Fig. 1.2).

In both R. acidophila and R. palustris reproduction is by the formation motile swarm cells. This is obligatory, and consequently these organisms have a dimorphic life cycle. Rm. vannieli exhibits greater cellular complexity and has a polymorphic growth cycle, the vegetative mother cells giving rise to either attached mother cells, swarm cells or exo-spores.

R. acidophila has the simplest life cycle of the budding purple non-sulphur bacteria (Pfennig, 1969) (Fig. 1.3). The cells are rod shaped or ovoid, 1.0 - 1.3 μm wide and 2 - 5 μm long, and rosettes or clumps of polarly aggregated cells are often observed. The swarm cells, which differentiate into mother cells, are ovoid and motile by means of subpolar flagella bundles (Tauschel and Hoeniger, 1974). During the development of a swarmer the flagella are shed and holdfast material is secreted at the same pole. A complex internal membrane system develops, probably as a result of the cytoplasmic membrane invaginating and folding back on itself repeatedly. Holdfast development is followed by polar growth and the formation of a sessile bud, which develops a flagella bundle and gives rise at division to a motile swarm cell. Consequently, at division two morphologically dissimilar cells, one bearing a holdfast and the other flagella, are formed.

The sequence of events in R. palustris are similar to those described
for R. acidophila, but of slightly increased complexity (Whittenbury and
McLee, 1967; Westmacott, 1975). A tube of slightly smaller diameter
than the cell body develops at the pole opposite the site of holdfast
formation (formerly the site of flagella attachment). Subsequent
daughter cell formation (ovoid, polarly flagellated swarm cells) occurs
at the end of this structure by budding. The temporal sequence of events
is described in Fig. 1.4.

One of the most complex models available for studying morphogenesis
and differentiation in procaryotes, and the subject of this thesis, is
Rhodomicrobium vannielii, a purple non-sulphur bacterium, which
exhibits a polymorphic life cycle (Dow, 1974; Whittenbury and Dow, 1977)
(Fig. 1.5).

Early reports of the morphological and developmental characteristics
presented a complex and ambiguous picture. This organism, which
reproduces by budding in the same way as the non-photosynthetic
Hyphomicrobium, was first isolated and described by Duchow and Douglas,
(1949). On the grounds of the apparent morphological similarities to
Hyphomicrobium (van Niel, 1944), it was classed in the family
Hyphomicrobiaceae, under the name of Rhodomicrobium vannielii (Duchow
and Douglas, 1949; Douglas, 1957). Physiologically, however, these two
organisms are quite distinct. Hyphomicrobium is an aerobic chemo-
organotroph whereas Rhodomicrobium is an anaerobic photoorganotroph.
Differences in gross morphology and ultrastructure also exist. Consequently
Rhodomicrobium is now classed in the family Rhodospirillaceae. (Pfennig
and Truper, 1971), formerly the Athiorhodaceae.

The reproductive mechanism of Rm vannielii and the accompanying
nuclear changes were first described by Murray and Douglas (1950).
Motility in this organism was not observed until Douglas and Wolfe (1959)
found that substituting sodium lactate for ethanol as the carbon source not
only increased the growth rate, but gave rise to coccoid peritrichously
flagellated cells in young cultures. Their development and role in the
growth cycle was not established at that time. Gorlenko (1969)(Gorlenko et al.(1974)
observed that Rhodomicrobium K 1, a strain physiologically and morpho-
logically similar to Rm vannielii forms large numbers of unattached
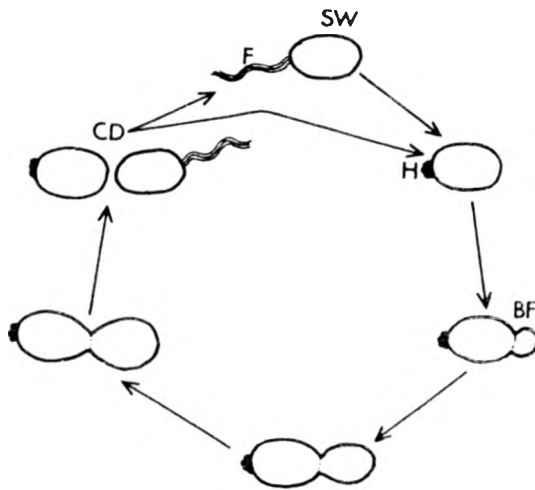
Fig. 1.3

Diagrammatic representation of the cell cycle of R. acidophila.
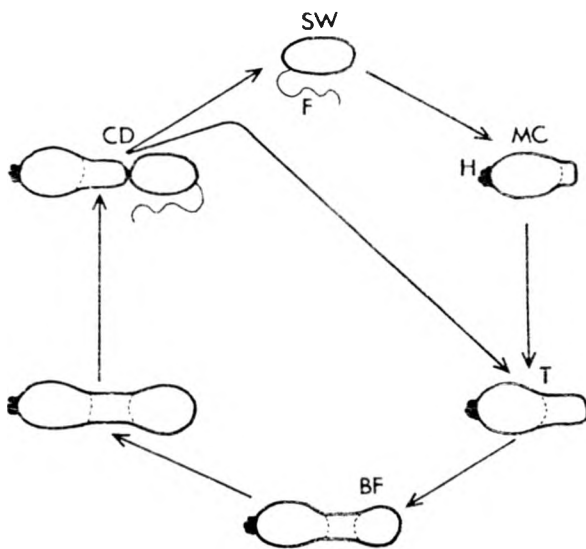
Fig. 1.4

Diagrammatic representation of the cell cycle of R. palustris.

LEGEND	SW	Swarm cell	BF	Bud formation
	F	Flagellum (flagella)	CD	Cell division
	MC	Mother cell	T	Tube synthesis
	H	Holdfast		
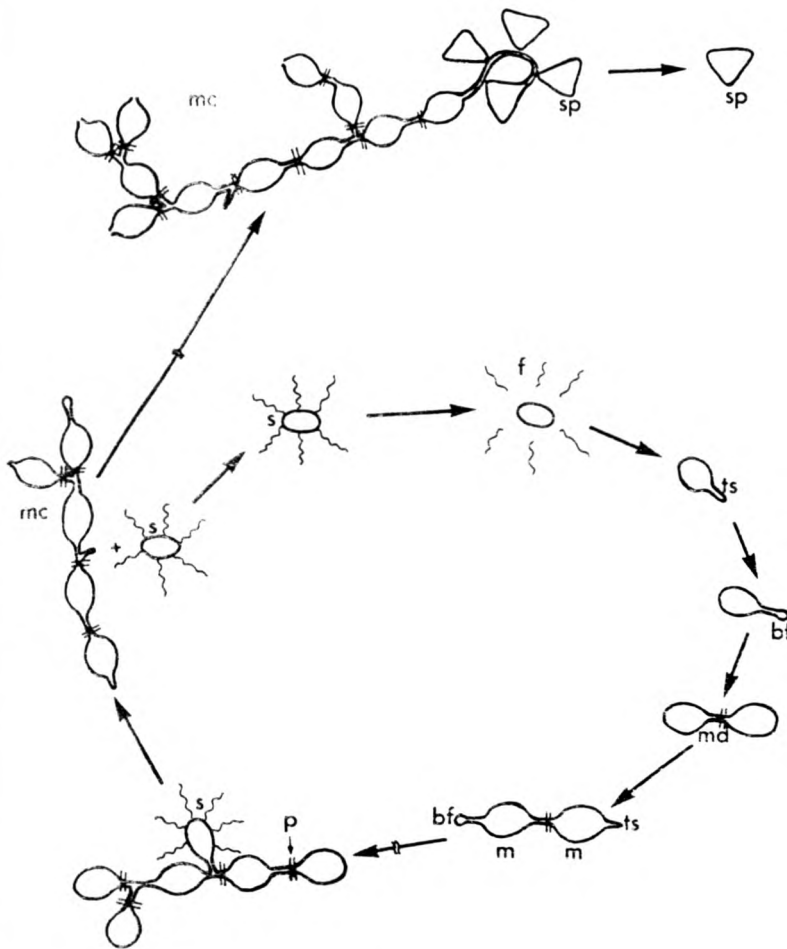
Fig. 1.5

Diagrammatic representation of the Rhodomicrobium cell cycle.

LEGEND

mc	multicellular array (microcolony)	
sp	exospore	
s	swarm cell	
f	flagella	
bf	bud formation	
ts	tube synthesis	
m	mother cell	
d	daughter cell	
p	cross wall 'plug'	

angular cells which, because of their optical properties, their reduced permeability to dyes and their thermostability, he called exospores. [Exospore and cyst production by budding from a mother cell has also been observed in two species of methane oxidising bacteria (Whittenbury et al., 1970)]. The production of these resting cells by Rm vannielii both anaerobically in the light and aerobically in the dark in a stationary phase culture, has been recently reported by Gorlenko et al., 1976.

As well as the morphological changes in Rm vannielii, many of the ultrastructural features have also been established (Boatman and Douglas, 1961; Conti and Hirsch, 1965; Trentini and Starr, 1967). More recently however, a considerable amount of work has been performed on Rm vannielii, and a new isolate designated Rm 5, which is indistinguishable from Rm vannielii (Dow, 1974). Rm 5 is the subject of this thesis, and will be described fully later. At present it is sufficient to say that it makes an excellent model system for the study of cellular morphogenesis and differentiation (Whittenbury and Dow, 1977). Obligate differentiation patterns are apparent, but in addition Rhodomicrobium also possesses the ability to undergo at least two forms of inducible differentiation, swarm cells and exospores, which is unusual and very appealing. Environmental control of this inducible differentiation has been implicated. In particular, nutritional control of swarm cell production by Rm vannielii was implied since motile swarm cells were not observed when cultures were grown on medium containing ethanol as carbon source (Duchow and Douglas, 1949), but appeared when this was replaced by sodium lactate (Douglas and Wolfe, 1959). Such evidence however is at best circumstantial. More recently, Gorlenko et al. (1976) have stated that any deviation of environmental conditions from optimal (increase of light intensity, changes in pH, aerobic dark cultivation) led to complete or partial inhibition of motile swarm cell production, but that such conditions stimulated sporulation. In earlier work, Gorlenko (1969) also suggested that other probable factors affecting the formation of specialised cells are substrate concentration, accumulation of metabolic products and population density. Clearly, the factors responsible for the induction of specialised cells in Rhodomicrobium have not been pinpointed ! Closer and more exacting investigations are warranted.

(iii) Environmental control of differentiation

Investigations of the genetic control mechanisms which operate in microbial cells indicate that environmental factors can, and do, influence gene expression (Zubay et al., 1970; Travers et al., 1970). The stimulation of differentiation by environmental factors has been investigated in a wide range of procaryotes and eucaryotes. Knowledge is limited, and in most cases the identification of the actual external factors responsible has proved difficult and elusive. Often it is impossible to distinguish between 'cause' and 'effect'. When one considers the influence of the environment on cellular expression, the process of sporulation immediately springs to mind. Spore formation is looked upon as a primitive form of cellular differentiation, and it has several features in common with cellular development in higher organisms.

The biological role of endospores is still unknown, but speculations have not been lacking. Many of the earlier suggestions have been reviewed by Knaysi (1948), Foster (1956) and Murrell (1961). One interpretation is that the cell recognises an unfavourable environment and in order to survive, it forms an endospore. It has also been concluded that it is extremely likely that spores represent a stage in the life cycle that allows certain bacteria to bypass unfavourable conditions (Cook, 1932). Objections to this role have been raised and modifications have been presented (Knaysi, 1948; Bisset, 1950) and discussed (Murrell, 1961; Lamanna, 1952). Bernlohr and Leitzmann (1969) have suggested that the mechanism just serves to put the cell into a new environment. Since growth cannot take place where the spore is formed, sporulation allows the cell to wait for more favourable conditions or to be transported to them and this gives it a selective advantage. Three roles assigned to spores (Sussman and Halvorsen, 1966; Sussman, 1969) and generally accepted can be summarised as enhancement of survival potential, disseminability and co-ordination of development when favourable conditions return.

Two external factors considered to be the most likely inducers of sporogenesis are:-

(a) The reduction of the level of growth supporting substrates which repress sporulation; glucose depletion, (Schaeffer et al., 1965; Dawes and Mandelstam, 1970), phosphate limitation (MacKechnie and Hanson, 1968) or amino acid starvation (Holmes and Levinson, 1967). The regulatory role of these compounds (e.g. glucose depletion derepresses sporulation) by catabolite repression will be discussed later.

(b) The accumulation of catabolites; a low-molecular weight ninhydrin-negative sporulation factor (produced in the presporulation period) has been isolated and purified from B. cereus and B. subtilis. The addition of this factor to vegetative cultures may induce sporogenesis (Srinivasan and Halvorsen, 1963; Srinivasan, 1966). An unknown factor is excreted into the medium by exponentially growing Clostridium butyricum which diminishes the growth rate and induces sporulation (Bergère and Hermière, 1965).

Nutritional control of cellular differentiation has been demonstrated in Azotobacter vinelandii. Encystment, the production of metabolically dormant resting cells, similar to spores, can be induced by using n-butyl alcohol, crotonate or β-hydroxybutyrate as carbon sources (Lin and Sadoff, 1968; Hitchins and Sadoff, 1970). Calcium ions are also believed to stimulate this change of cellular expression (Page and Sadoff, 1975).

The Coryneform bacterium, Arthrobacter crystallopoietes, also exhibits cyclic morphological variations (coccus/rod)/(Fig. 1.6). (Lucas and Clark, 1975) Ensign and Wolf (1964) developed a simple chemically defined medium that restricted growth to the coccoid form. Addition of L-arginine, L-phenylalanine, L-asparagine, L-lysine, succinate, malate, fumarate or butyrate resulted in the formation of a rod shaped stage. Luscombe and Gray (1971), however, demonstrated, using continuous culture techniques, that this transition was a direct result of the change in growth rate and not specifically due to the presence of an environmental trigger compound. Duxbury and Gray (1977) have observed that this organism may be induced to form enlarged, oval or lemon shaped cells, called cystites, by growth in medium containing a very high carbon to nitrogen ratio.

Enteric bacteria have been studied with respect to their response to environmental conditions. Heavy metal ions at very low concentrations inhibit the motility of E. coli. Amino acids and compounds such as ethylene diamine tetra acetic acid (EDTA), peptone, albumin, mercapto-ethanol and oxalate to name but a few, remove the inhibition and stimulate motility by chelating the heavy metals (Adler and Templeton, 1967). The behaviour of Proteus on agar plates is particularly interesting (Hoeniger, 1964; Jones and Park,1967a;Bisset,1950,1973;Morrison and Scott, 1966). It is commonly believed that the formation of concentric zones of growth around a point of inoculation by swarming strains of Proteus is due to a rhythmic alternation of elongated motile swarming bacteria with shorter, non-swarming ones at the leading edges of the swarm colony. The accepted physiological explanation is that swarming is a device to escape from concentrations of toxic metabolites produced by growth of the sessile stage, and that the former reverts to the latter when the stimulus is removed (Hughes, 1957; Grabow, 1972). Jones and Park (1967b) investigated the influence of medium composition on the growth and swarming of Proteus. Swarming did not occur on minimal medium agar unless alanine, asparagine, aspartic acid, glutamic acid, glutamine, proline or serine were present either individually or together. They therefore concluded that the ability of a compound to support swarming was correlated with its ability to serve as carbon-energy source and with the stimulation of growth rate.

The phenotype of the morphologically unusual Ancalomicrobium, first described by Staley (1968), varies considerably according to the nutrient status of the growth medium. Under conditions of low organic nutrients (50 µg/ml and less) the cells exist as multi-appendaged prosthecate bacteria,whereas under conditions of high organic nutrients (200 µg/ml and greater) they appear as distinctive rods (Fig. 1.7) (Dow, 1974; Dow and Lawrence, 1977).

In the gliding bacteria Myxococcus xanthus and Myxococcus virescens nutritional control of cellular differentiation has also been established (Dworkin and Gibson, 1964;/Parish et al., 1976). Both organisms are typical myxococci and on solid media with low concentrations of nutrient
Dworkin and Sadler, 1966;

they can form fruiting bodies within which the vegetative cells differentiate to become myxospores (Fig. 1.8). Such conversion can be rapidly and synchronously induced in liquid culture by glycerol and several alcohols (Dworkin , 1962; Dworkin and Sadler, 1966). The presence of the divalent cations Mg^{++} and Ca^{++} is essential. The process is reversible by the removal of the inducer and will only take place with exponentially growing cells. Myxospore formation in Stigmatella aurantica, another member of the Myxobacteriales, can be induced by the addition of mono-valent cations such as Li^+, Na^+, NH_4^+, K^+ and Rb^+ to suspension cultures (Reichenbach and Dworkin, 1970).

Environmental variables seem to exert a pronounced effect on the behaviour of the complex multicellular procaryotes of the Actinomycetes and this topic has been recently reviewed by Kalakoutski and Agre (1976). Geodermatophilus (Luedemann , 1968) grows in two forms, as a non-motile, irregularly shaped aggregate of coccoid cells (C form) and as a dimorphic budding rod (R form) (Fig. 1.9). Morphogenesis can be controlled by a factor in tryptose which is required for maintenance of the organism in the C form and for differentiation of the R form to the C form (Ishiguro and Wolfe, 1970). Subsequently, during work attempting to identify the tryptose factor, it was found that a variety of inorganic cations (Ca^{++}, Mg^{++} and organic nitrogenous cations) were able to control morphogenesis (Ishiguro and Wolfe, 1974). The latter were shown to raise the intracellular pH of the cells and it was suggested that this enhanced a metabolic event necessary for cellular differentiation. Roberts (1964), working on Dermatophilus dermatonomus, showed that CO_2 affected both the growth of the actinomycete and the movement of its zoospores. The addition of sodium and potassium salts to the cultures potentiated the effect of CO_2 in stimulating hyphal growth and delaying sporulation. One further example of the effect of complex nutrient additives is given in studies with Nocardia sp 721-A, which forms clublike cells on medium with meat extract. These cells, which undergo irregular septation, do not appear on nutrient agar and mineral synthetic media with glucose or saccharose (Beaman and Shankel, 1969). Sporulation of Streptomyces is believed to be under environmental control and enhanced on starvation media containing mineral rather than organic sources of nitrogen (Schneidau and Schafter, 1957; Gordon and Mihm, 1957).
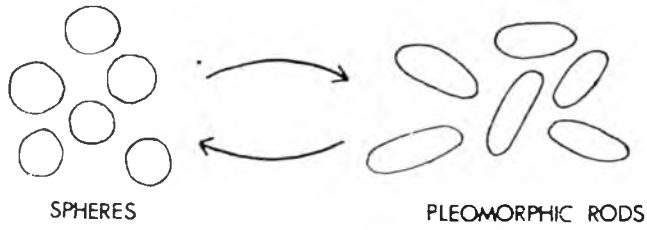
Fig. 1.6

Phenotypic variation associated with Arthrobacter crystallopoietes
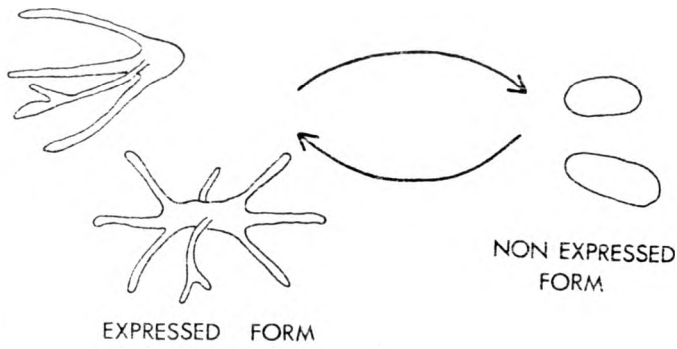
Fig. 1.7

Phenotypic variation associated with Ancalomicrobium
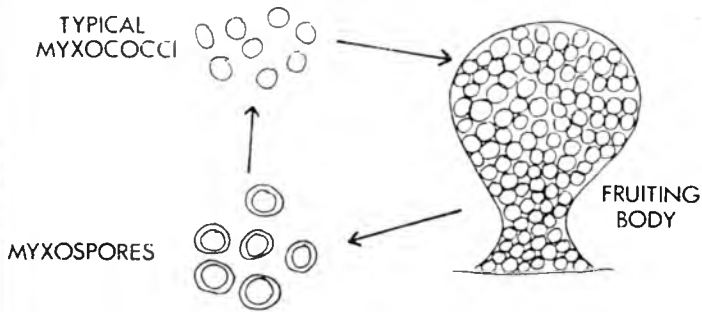
Fig. 1.8

Phenotypic variations associated with Myxococcus
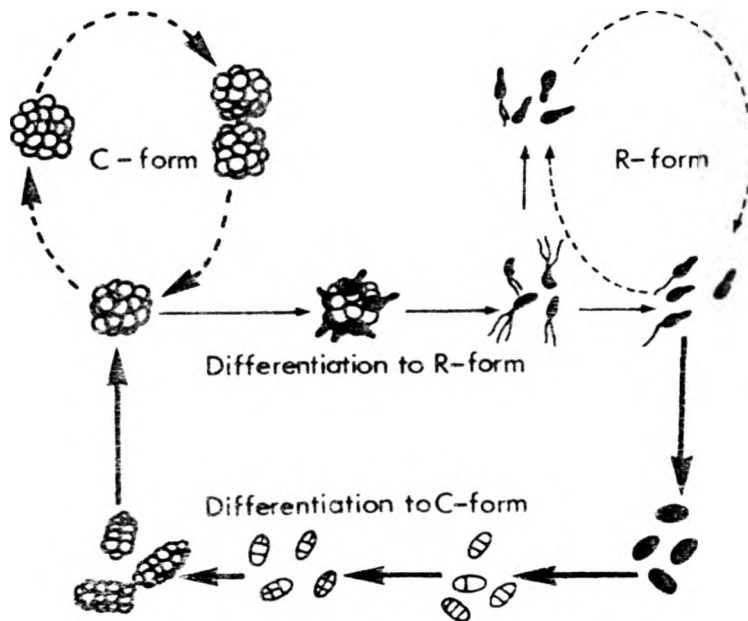
Fig. 1.9

The polymorphic growth cycle of Geodermatophilus strain 22-68 (after Ishiguro and Wolfe, 1970).

Maintenance of the C form requires the presence of a factor found in tryptose. Absence of this factor induces differentiation to the R form. Monovalent and divalent inorganic cations (Na^+, NH_4^+, Ca^{++}, Mg^{++}) as well as organic nitrogenous cations (methylamine hydrochloride) induce R form to C form morphogenesis and maintain the organism in the C form.

The morphological cell types and complexity of life cycles in the filamentous cyanobacteria (blue green algae) are of the most diverse amongst procaryotic organisms. The major differentiated cell types that occur are akinetes (resistant cells) and heterocysts (specialised cells believed to be the sites of nitrogen fixation). The effect of environmental conditions on the morphology of the cyanobacterium Chlorogloea fritschii has been studied (Evans et al., 1976) and it was found that the availability of reduced carbon substrate, light, nitrogen and temperature all caused alteration in cellular expression. Here the two major cell types were irregular clumps of cells (aseriate) and filaments (Fig. 1.10). In photoautotrophic conditions, the former predominated during exponential growth at 34° C. The presence of sucrose imposed aseriate morphology in both phototrophic and hetero-trophic cultures. The development of heterocysts followed deprivation of nitrate. It is thought that the different cell types observed are better described as the products of particular environmental conditions than as stages in an order of developmental sequence. Heterocyst formation in Anabaena cylindrica is induced when cultures are deprived of fixed nitrogen (Fogg, 1949). More recently, however, Bradley and Carr (1977) have demonstrated the necessity for light as an initial trigger for heterocyst development in this organism.

Fungi, which exist in two morphological forms, provide a popular and useful system in which to study the biochemical basis for vegetative differentiation, the nature of the the change and its regulation. The environmental influence amongst these simple eucaryotes, and in particular yeast, has been widely documented (Bartnicki-Garcia and McMurrough, 1971). Spontaneous morphological changes occurring within a yeast colony over a solid substrate usually results from modifications in the environment caused by the yeasts themselves. When Candida albicans is grown on solid medium with glucose as sole carbon source, colonies are initially composed of typical yeast cells which reproduce by forming buds. (The buds enlarge until they are almost equal in size to the mother cell, nuclear division occurs and then a cross wall is formed between the two cells). Later, however, extensive filamentation (highly
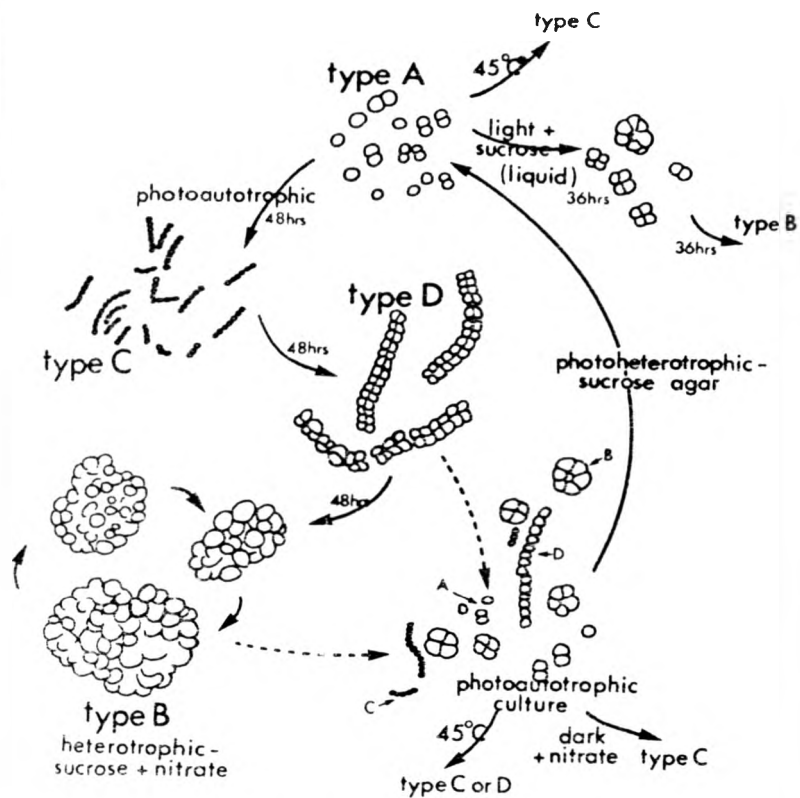
Fig. 1.10

Diagrammatic summary of the morphological variations induced in
Chlorogloea fritschii by different environmental stimuli (after Evans et al.,
1976).

Morphological cell types:-

Type A: Large granulated cells (2 x 3μm) existing either singly or as
            clumps containing two or more cells which arise from division
            in up to three planes.

Type B: Aseriate, large irregular clumps surrounded by a mucilaginous
            sheath.

Type C: Short filaments of small (1 μm) cells.

Type D: Larger cells than type C (1.5 μm) and found in filaments in
            the process of dividing.

elongated cells and distinct hyphal forms) due to impairment of the division process, becomes apparent around the periphery of the colony (Fig. 1.11). This isproduced in response to alterations in the composition of the medium by the metabolic activities of the yeast itself. The substitution of a less readily utilizable carbon source to the medium, such as glycogen, results in filamentation from the beginning of cultivation (Nickerson and Mankowski, 1953). However, this can be prevented by the addition of sulphydryl (-SH) compounds such as cysteine, glutathione or thioglycollate to the medium. The stimulation of budding by -SH compounds was interpreted (Nickerson, 1948) as one further instance of the widespread requirement of the -SH group in the division processes of living cells. Reduction breakage of cell wall disulphide links causes plasticising of the cell wall favouring the yeast form of growth (Fig. 1.12). Two other observations provided additional support for the role of the sulphydryl-sulphide balance in the dimorphism of Candida albicans. Addition of selenite or tellurite, both of which can be incorporated into cell wall proteins, caused a filamentous mutant of C. albicans to grow yeast like. It has been suggested that because the selenhydryl or tellurhydryl groups are more stable than sulphydryl groups, they offset the deficiency of protein disulphide reductase present in the the filamentous mutant (Nickerson et al., 1956). It was also observed that when the filamentous mutant was grown in sulphur deficient medium, its cellular content of sulphur diminished and its development was mainly in the yeast form. It was suggested that under these conditions relatively few disulphide cross links would be formed in the cell wall and the low level of protein -disulphide reductase present would be sufficient to produce the degree of cellular plasticity compatible with budding (Falcone and Nickerson, 1959).

Control of dimorphism in Candida albicans by zinc has also been observed (Widra, 1964). Zinc deficient cultures consist almost entirely of 'filamentous' cells but the proportion of 'yeast' cells increases with increasing concentrations of zinc. The participation of zinc in this way has also been observed in Histoplasma capsulatum (Pine and Peacock, 1958)

YEAST FORM   FILAMENTOUS FORM
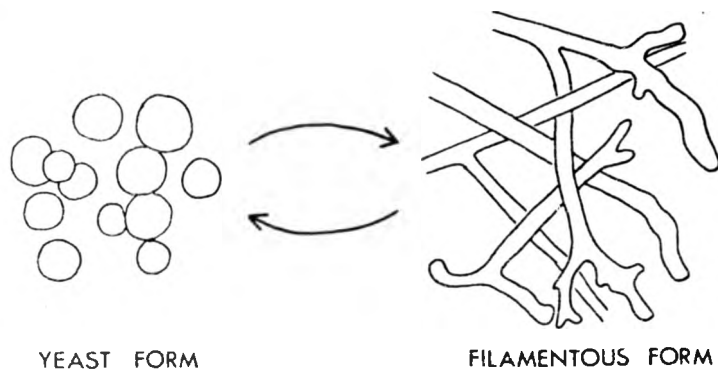
Fig. 1.11

Yeast and filamentous expression of Candida albicans.   Similar phenotypic
variation is associated with Mucor rouxii.

RIGID CELL WALL FABRIC   DEFORMABLE CELL WALL FABRIC
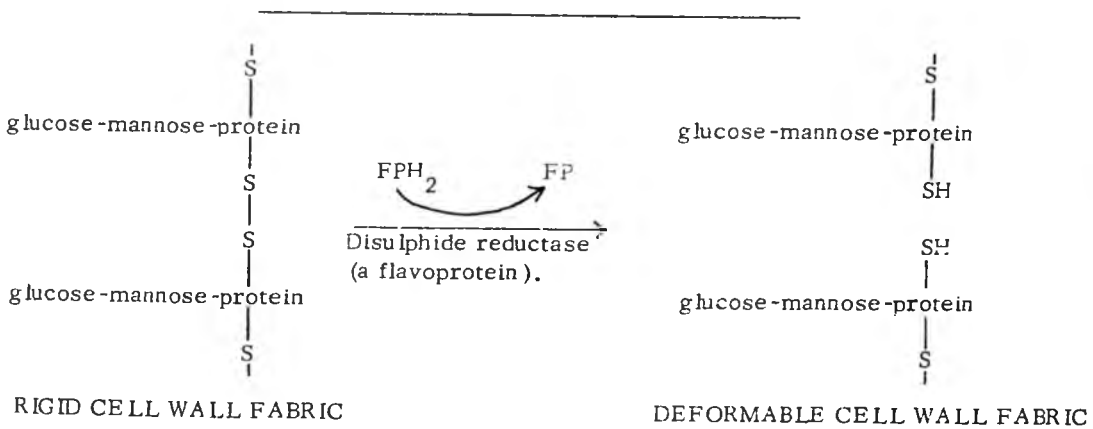
Fig. 1.12

Biochemical action of disulphide reductase on cell wall fabric (after
Bartnicki-Garcia and McMurrough, 1971).

and Mucor rouxii (Bartnicki-Garcia and Nickerson, 1962b). It has also been suggested (Yamaguchi, 1975) that zinc has a primary function in the metabolism of RNA in C. albicans rather than protein or DNA. Zinc may be required for the synthesis of RNA or in preventing its degradation. Whatever the mechanism of action may be, the complete cessation of net RNA synthesis at the transitory stage of 'yeast' to 'filamentous' growth under a certain degree of zinc deficiency implies that subsequent filamentous elongation takes place without net ribosomal formation. There is, therefore, a significant difference in the metabolic processes involved in mycelial elongation ('filamentous' growth) and simple cell division ('yeast' growth).

Numerous factors including temperature, nutrition, age, pH and the presence of certain chemicals in the medium are known to affect the elongation of typical yeast cells such as Saccharomyces cerevisiae which manifest only a limited capacity for cell elongation (Scherr and Weaver, 1953). Fusel oils have also been implicated as morphogenetic inducers (Geiger, 1961; Grant, 1964). Elongation also occurs in continuous culture with defined medium when the concentration of nitrogen (supplied as ammonium sulphate, methionine or asparagine) becomes limiting (Brown and Hough, 1965; McMurrough and Rose, 1967). The addition of sodium thioglycollate or selenate, however, effects a reversion of the elongated cells back to ellipsoidal cells. It was concluded, in analogy with the observations on C. albicans, that the morphological effect was due to an alteration in the cellular balance of the sulphydryl-disulphide.

Another pattern of yeast morphogenesis is displayed by Trigonopsis variabilis where there is an interchange between a triangular cell shape and the conventional yeast form, (Fig. 1.13), which is dependent on environmental conditions. Formation of triangular cells is stimulated by the availability of a suitable methyl donor in the growth medium, i.e. methionine or choline (Senthe Shanmuganathan and Nickerson, 1962). The determinant role of the methyl donor substrates in triangular cell morphogenesis was questioned by Sasek and Becker (1969) who found that proline, alanine and hydroxyproline were equally effective in promoting triangular cell morphogenesis. They also found that triangular cell development principally occurred during the early log phase and not
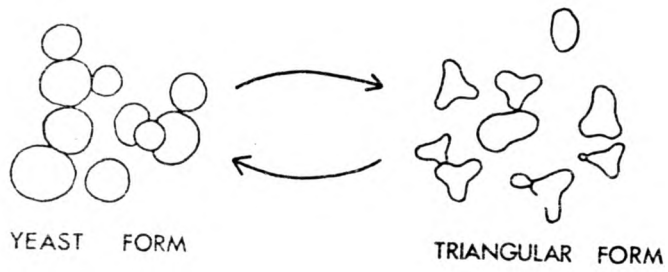
Fig. 1.13

Phenotypic variation of Trigonopsis variabilis

during the stationary growth phase. This indicated that environmental conditions conducive to triangular cell morphogenesis were transient.

Possibly one of the most widely studied simple eucaryotes is the dimorphic fungus Mucor. Mucor rouxii and other species are typical mycelial fungi endowed with the potential to develop into populations composed mainly or exclusively of budding spherical yeast cells (Fig. 1.11) (Bartnicki-Garcia, 1963). Vegetative morphogenesis has been shown to depend on a variety of environmental factors:-

(a) Oxygen Under anaerobiosis, Mucor rouxii spores germinate producing yeast like cells which reproduce by budding, whereas aerobic development leads to the formation of a typical mycelium (Bartnicki-Garcia and Nickerson, 1962a).Similarly Haidle and Storck (1966) were able to convert the yeast cells of M. rouxii growing on defined medium to the mycelial form by changing the gas phase of the culture atmosphere from carbon dioxide /nitrogen or nitrogen to air.

(b) Hexoses Rogers et al. (1974) concluded that glucose was the most important single factor determining the morphological state of M.genenvensis. Using continuous culture techniques it was observed that under conditions of glucose limitation, oxidative metabolism increased as the dissolved oxygen concentration increased, resulting in an increase in the proportion of the mycelial form in the culture. Filamentous growth was inhibited by glucose, which induced a complete reversion to the yeast-like form. The effect of glucose on the germination of spores of M. rouxii was studied by Friedenthal et al. (1974).At 0.01%(w/v) glucose concentration mycelial development occurred, whereas at increasing concentrations dimorphic colonies were formed. 2% (w/v) glucose resulted purely in the appearance of the yeast form. Similar effects were observed with fructose and mannose (Bartnicki-Garcia, 1968).

(c) Carbon dioxide This has been widely documented as able to regulate dimorphism in Mucor. Elmer and Nickerson (1970) found that carbon dioxide at $p\,CO_2$ values of 0.3 atm or higher induced yeast-like development in 5 strains of M. rouxii whereas growth under air or nitrogen was filamentous. Certain nutritional factors including a small

peptide, however, needed to be present. Bartnicki-Garcia and Nickerson (1962a) had similarly observed that under nitrogen filamentous growth of M. rouxii occurred followed by fragmentation of the hyphae into arthrospores. Introduction of carbon dioxide induced spherical budding yeast cells but this effect was nullified by the presence of oxygen.

(d) Other factors A large number of other parameters have been observed to effect 'yeast' to 'filamentous' morphogenesis or vice versa. These include:- phenethylalcohol (Terenzi and Storck, 1969), an unidentified volatile fraction (Mooney and Shepherd, 1976), cyanide, acriflavin and cyclohexamide (Haidle and Storck, 1966) and chloramphenicol (Clark-Walker, 1973; Friedenthal et al., 1974). Heavy metals and dicarboxylic acids have also been implicated (Bartnicki-Garcia and McMurrough, 1971).

It would be misleading, however, to assign any one of these factors the causal role of dimorphism, since the effect of each factor is conditioned by the concentration of the others. For example, in anaerobic cultures the control of morphogenesis in M. rouxii by carbon dioxide depends on the hexose concentration (Bartnicki-Garcia, 1968). (In vivo CO_2 production from the hexose is a possibility). A high $p\,CO_2$ value generally favours the development of yeast cells but if the hexose concentration is below $0.1\%\,(w/v)$, no yeast development occurs even under an atmosphere of 100% carbon dioxide. Conversely, if the glucose concentration is above $8\%\,(w/v)$, exogenous carbon dioxide is no longer a requirement for yeast development. This interaction between hexose and carbon dioxide concentration is further conditioned by the concentrations of other factors (Bartnicki-Garcia, 1968), which was attributed to dialysable factors by Elmer and Nickerson (1970).

Speculative mechanisms have been suggested to explain the complex interactions of environmental factors on yeast morphogenesis which depend on the intracellular concentrations of certain metabolites (Bartnicki-Garcia and Nickerson, 1962b). Alternative mechanisms which need not be mutually exclusive have been proposed (Bartnicki-Garcia, 1963) which involve interference of the assembly of cell wall proteins (Bartnicki-Garcia and Lippman, 1969). Seemingly environmental factors which interfere

with the establishment of polarized wall synthesis, cause the cell wall to grow uniformly over its entire periphery, giving rise to spherical yeast cells.   Strong support has been given to the suggestion by Terenzi and Storck (1969) that the filamentous morphology in Mucor might be regarded as morphological expression of the Pasteur effect, i.e. yeast-like morphology and fermentation are linked, and filamentous-like morphology and respiration are linked, and that conversion of the filamentous form to yeast form by phenethyl alcohol is due to the inhibition of oxidative phosphorylation.   The work done by Clark-Walker (1973), however, suggested that the view that phenethyl alcohol promotes yeast-like morphology because it stimulates fermentation and uncouples phosphorylation should be re-examined.   He observed that, although chloramphenicol eliminates functional mitochondria, it does not lead completely to the yeast-like form.

As a final illustration, environmental control of differentiation has been widely implicated in the acellular slime moulds. Differentiation in Dictyostelium discoideum has been widely reviewed (Sussman and Sussman, 1969; Ashworth, 1971; Garrod and Ashworth, 1973; Killick and Wright, 1974).   This organism grows in the soil as a population of independent uninucleate amoebae. With the onset of the stationary phase, the amoebae aggregate to form organised multicellular fruiting bodies composed of spores and stalked cells (Fig. 1.14).   Depending upon the environmental conditions, the developing aggregate either constructs the fruiting body at the site of aggregation or transforms into a structure known as a slug which can migrate to a more favourable location. Newell et al. (1969) suggested factors which favour migration to be

(a) accumulation of metabolites produced by the aggregate,

(b) low ionic strength of the substratum.

Conditions preventing migration or stopping a migrating slug are

(a) presence of buffer,

(b) illumination by overhead light.

The effect of other factors such as ammonia, and humidity on morpho-genesis in this organism, as well as light and temperature, have also been described by Bonner et al., (1950), Bonner and Shaw, (1957), Cohen (1953) and Francis (1964).   The responses of a developing slime mould to a number of environmental influences appear not to be arbitrary but can be seen as most suited for survival and propagation under the circumstances available.
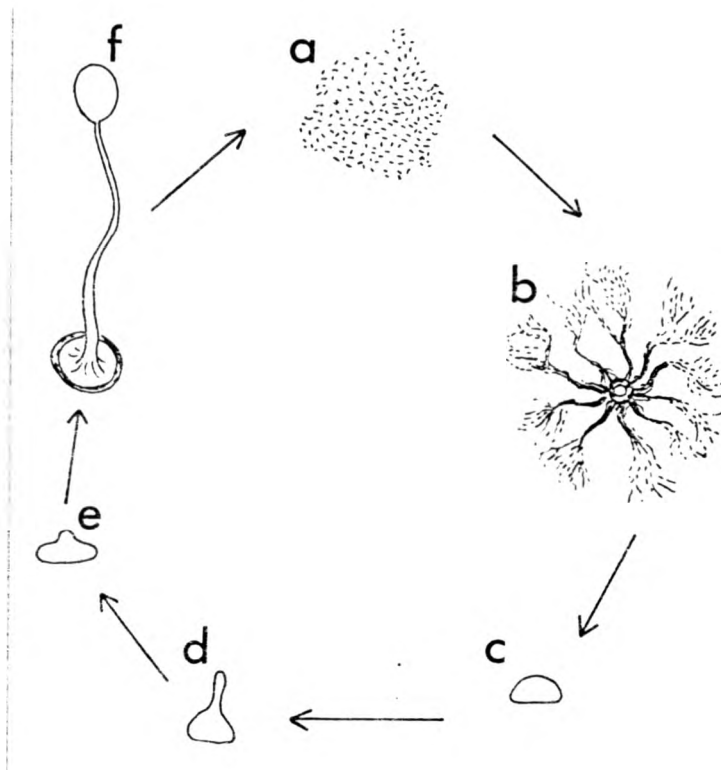
Fig. 1.14

Diagrammatic representation of the life cycle of Dictyostelium discoideum

(a)   Uniform mass of vegetative amoebae.

(b)   Aggregation.

(c)   'Slug' formation.   Migration.

(d)(e)   Early stages in the formation of a fruiting body.

(f)   Mature fruiting body.

(iv) Regulation of differentiation at the molecular level

The purpose of this introduction so far has been to describe the morphological changes that occur in a number of systems currently used as models of cellular differentiation, and to outline the possible role played by the environment in controlling this expression. A brief discussion of the possible regulatory phenomena, at the molecular level, which control cell cycle expression is also justified.

Cell division, which is differentiation at the simplest and most basic level, is common to all developmental systems. The most detailed studies have been carried out on E. coli, (Donachie et al., 1973: Pardee, 1974; de Pedro et al., 1975; Meacock and Pritchard, 1975; Ron et al., 1977; de Pedro and Canovas, 1977, are but a few) and to a lesser extent on B. subtilis (Donachie et al. 1971) and Salmonella typhimurium (Shannon et al., 1972). To ensure that each daughter cell receives a copy of the genome DNA replication, protein synthesis and cell division must be precisely timed and co-ordinated. It has been suggested (Jones and Donachie, 1973) that the DNA replication and cell division cycles are controlled by two separate timing systems or 'clocks' and that both are probably initiated at the same time in response to the cell doubling its initiation mass. Completion of cell division is dependent on both clocks and presumably therefore, on the combined products of the two cycles.

The length of the chromosome replication cycle in E. coli at constant temperature remains remarkably constant for a variety of growth rates (Cooper and Helmstetter, 1968). The replication rate is governed by the number of initiation points and not the rate of nucleotide addition. Dichotomous replication has been shown to occur in B. subtilis (Suecka et al., 1964) as well as E. coli (Cooper and Helmstetter, 1968; Masters, 1970) and it is believed to be universal. At different growth rates (with the exception of very slow growth rates) the rate of addition of nucleotides to the replication fork remains constant; however, new rounds of chromo-some replication are initiated at every doubling in cell mass (i.e. the faster the growth rate the greater the number of DNA initiation points).

The nature of the division clock is unknown. Experiments on the necessity of prior protein synthesis for division (Pierucci and Helmstetter, 1969) however, strongly suggest that a constant period of protein synthesis, of about 40 min, is required before division can take place. The fact that this time period is constant irrespective of growth rate indicates that this is part of the time clock.

Once the initiation of cell division has been triggered, the genes coding for the cell division proteins can be transcribed. To prevent the formation of anucleate cells it has been shown in E. coli that completion of a round of replication is a necessary pre-requisite for division (Clark, 1968; Pierucci and Helmstetter, 1969). This may be to allow segregation of the daughter chromosomes, which is mediated by the synthesis and growth of new membrane from an equatorial region between their attachment sites and hence provide space for a septum to form between them (Jacob et al., 1963). Alternatively it may be that at termination a late gene essential for cell division is transcribed and that this late gene is unusual in so far as it is only transcribed when replicated. Evidence has been obtained (Jones and Donachie, 1973) that the transcription of a specific gene (or genes) is required if cell division is to be completed, and that this transcription takes place at or immediately after termination of each round of chromosome replication. Enough of the termination protein(s) can be made in 5 min to allow division to take place (Donachie et al., 1973; Jones and Donachie, 1973). It has been suggested that the termination proteins are components of the cell envelope involved in the dissociation of the chromosome from the old replication complex into a septum (Donachie, 1969; Donachie and Begg, 1970). Elucidation of the regulation processes employed in cells with simple life cycles whose only major differentiation step is cell division have proved difficult and they are still not fully understood. However, a large number of procaryotes, both bacteria and cyanobacteria, have cell cycles complicated by temporal and inducible differentiation of subcellular organelles and altered cell forms. The increased demand on the regulatory machinery is immense, but knowledge on how the cells are able to cope is emerging. Particular

interest has been shown in endospore formation and outgrowth in Bacillus. Enzyme activities in the spore are, however, different from those found in the vegetative cell, and this change is believed to be brought about by the suppression of some vegetative genes during sporulation, and the sequential expression of new genes. A rapid loss of activity of at least 7 enzymes (lysine decarboxylase,isocitrate dehydrogenase, threonine dehydratase, aspartokinase, aspartate transcarbamylase,inosine mono-phosphate dehydrogenase and diaminopimelate decarboxylase) occurs during the initiation of sporulation in several species of Bacilli (Bernlohr and Gray, 1969; Deutscher and Kornberg, 1968). In contrast, new proteins obviously needed for normal sporulation are dipicolinate synthase (Chasin and Szulmajster, 1969; Bach and Gilvarg, 1966; Fukuda et al., 1969) and coat proteins that comprise up to 80% of the spore protein (Aronson and Fitz-James, 1968; Aronson and Horn, 1969). Proteasesare also produced in vast amounts during the sporulation phase of Bacilli (Levisohn and Aronson, 1967; Prestidge et al., 1971; Setlow, 1975) and at least one function of these is believed to be the processing of spore coat precursor proteins (Cheng and Aronson, 1977a). Most spore enzymes, however, are believed to be the product of the same genes which produced the vegetative cell counterparts (Nelson et al., 1969). Some of these enzymes are modified after their synthesis or have altered properties due to a conformation change. Such is the case with aldolase (Sadoff et al., 1970) which is changed from a heat sensitive to a heat stable enzyme during sporulation, by protease removal of a peptide from the vegetative form. Recently, however, proteases have been implicated in the modification of RNA polymerase (Millet et al., 1972; Lecadet et al., 1977; Cheng and Aronson, 1977a, b).The importance of this is outlined later.

Obviously the control of the sequential steps of sporulation are of great interest. Induction-catabolite repression of spore enzymes and catabolic enzymes is widely believed to occur (Laishley and Bernlohr, 1966; Coote, 1974) and it has been suggested that initiation of sporulation is triggered by low energy levels (Hutchinson and Hanson, 1974).

Schaeffer et al. (1965) determined the effect of various carbon and nitrogen sources on the commitment of B. subtilis to sporulation. They concluded that catabolite repression prevented the initiation of sporulation and that the effector contained nitrogen. Holmes and Levinson (1967) showed that nitrate limitation induced microcycle sporogenesis of B. megaterium whereas MacKechnie and Hanson (1968) observed that this phenomenon in B. subtilis resulted from phosphate limitation. These results were consistent with the hypothesis that catablic repression was occurring, and that the effector contained nitrogen and phosphate. As is the case for normal cell division, DNA replication and its completion is a requisite for successful sporulation (Mandelstam et al., 1971). Three models have been put forward to account for the sequential processes of sporulation, and these have been widely discussed (Mandelstam, 1969; Halvorsen, 1965; Waites et al., 1970).

(a)   Simple sequential transcription.

(b)   Sequential transcription mediated by sigma (σ) factors.

(c)   Sequential induction.

The first model, however, is unlikely as gene orders did not match the order of events, and it is now more widely accepted that transcriptional control may be a combination of the other two models. Bautz et al. (1969) have shown that after infection of E. coli by phage T4, there are changes in the specificity of the RNA polymerase, and that these are produced by proteins which they call sigma (σ) factors. Transcriptional control of cellular differentiation, due to change in template specificity either by modification of the existing RNA polymerase or the presence of a different polymerase, has been shown to occur in sporulating Bacilli and considerable evidence is accumulating (Losick and Sonenshein, 1969; Sonenshein and Losick, 1970; Losick et al., 1970; Doi et al., 1970; Linn et al., 1973; Murray et al., 1974; Linn et al., 1975, Fukuda and Doi, 1977). Recently, however, translational control of gene expression during differentiation in these cells has been implicated. Alterations of ribosomal proteins was observed during spore formation in B. subtilis, which alter the function of the

translation mechanism (Fortnagel and Bergmann, 1973). Specific alterations of the 30S ribosomal subunits of B. subtilis during sporulation have been observed by Guha and Szulmajster, (1977). Support for the third model has come from Piggot (1973) who demonstrated, using asporogenous mutants, that there are at least 28 operons associated with sporulation in B. subtilis and suggested that several a. e probably activated in groups. These operons are not together and probably scattered throughout the genome.

Transcriptional control of spore outgrowth as well as formation is likely, since hybridization studies have shown that individual species of RNA are synthesised periodically (Hansen et al., 1970). It seems probable that there is a gradual transition of expression of the genes during outgrowth. After germination, RNA synthesis occurs first, and this is closely followed by the onset of protein synthesis and finally DNA synthesis at a later stage. This protein synthesis is believed to be dependent on prior RNA synthesis (Sakakibara et al., 1965; Kobayashi et al., 1965) since there is no evidence for long lived messages.

Lately considerable attention has been focussed on the regulation of the complex Caulobacter cell cycle (Kurn and Shapiro, 1975). This organism bears a close relationship with the cell morphology of the budding photosynthetics and has been widely recognised as a suitable model for studying bacterial differentiation (Fig. 1.15). Kurn and Shapiro (1975) suggested that, in an analogous manner to B. subtilis, there are two programs for gene expression, one of which is expressed in the stalked daughter cell and carried through to cell division, and the other in the swarmer daughter cell. Experiments with inhibitors of DNA synthesis suggested that here, as in other procaryotes, DNA replication and completion of the genome was obligatory for cell division (Degnen and Newton, 1972a,b) .By studying the effect of rifampicin, which blocks the initiation of the RNA synthesis, Newton (1972) also demonstrated that cell division and initiation of DNA replication were dependent on de novo RNA synthesis, and that loss of swarm cell motility was delayed by inhibition of RNA synthesis. It was concluded that initiation of the
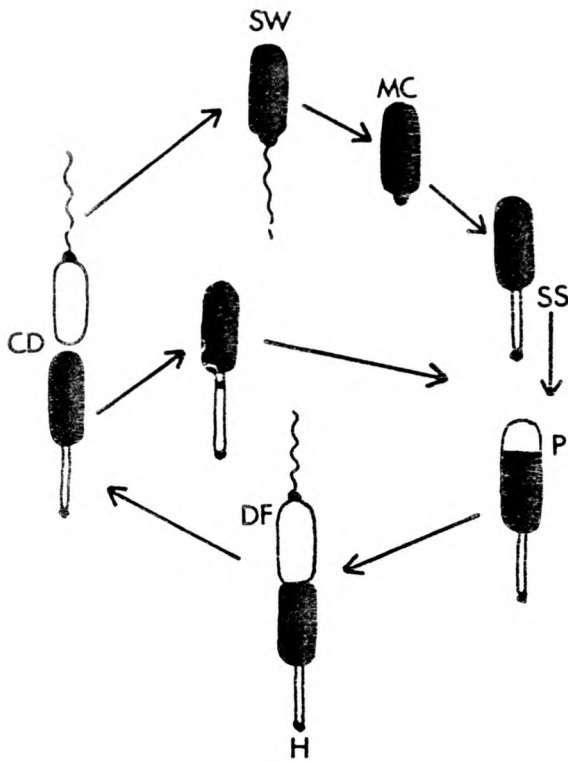
Fig. 1.15

Diagrammatic representation of the life cycle of Caulobacter crescentus

LEGEND
SW	Swarm cell
MC	Mother cell
SS	Stalk synthesis
P	Polar growth
H	Holdfast
DF	Daughter formation
CD	Cell division

morphological transitions was regulated, at least in part, at the level of transcription. In view of the findings with B. subtilis, it was suggested that this may be effected by altering the template specificity of the RNA polymerase. However, polyacrylamide gel electrophoresis analyses of RNA polymerases from various Caulobacter cell forms have indicated that morphological and biochemical changes associated with development cannot be accounted for by major structural changes in the RNA polymerase (Wood and Shapiro, 1975; Bendis and Shapiro, 1973). It is now thought that regulation, solely at the level of transcription, is unlikely. Analysis of ribosomes from different genera, and natural messenger RNA's from their respective phages, has indicated that regulation of Caulobacter expression also exists at the translational level (Shapiro, 1976). Studies with hybrid ribosomes have shown that the specificity of ribosome binding and translation of natural messenger RNA's is primarily a function of the 30S subunit (Lodish, 1970; Leffler and Szer, 1973). Evidence is accumulating that the components responsible for the specificity of messenger recognition are the 16S ribosomal RNA as well as the S12 ribosomal protein (Held et al., 1974; Goldberg and Steitz, 1974; Shine and Delgarno, 1975).

(v) Involvement of cyclic nucleotides in differentiation

The involvement of cyclic nucleotides in the regulation of procaryotic gene expression is fairly certain, and their functions are believed to be diverse and numerous (Rickenberg, 1974). However, the question which has yet to be satisfactorily answered is 'Are they a determinant or a product of the developmental programme?'

It has been suggested that cyclic adenosine 3', 5'-monophosphate (cAMP) together with cAMP receptor protein regulates the transcription of numerous operons which code for bacterial catabolic enzyme systems (Makman and Sutherland, 1965; Tan and Lipmann, 1969; Nielson et al., 1973). The role of cAMP as a mediator of catabolite repression in E. coli has also been suggested by Saier et al. (1975) and Epstein et al. (1975) since it was shown that the levels of this cylic nucleotide were dependent on the carbon source in the environment. It was inferred that regulation of cAMP levels allowed a bacterium to choose preferential carbon sources. The role of cAMP in E. coli has been recently reviewed by Pastan and Adhya (1976).

Morphogenetic effects associated with variation in cAMP levels have also been demonstrated. It has been found to be associated with the regulation of flagella synthesis, and hence motility in E. coli and Salmonella typhimurium (Yokota and Gots, 1970). Sphere-rod-sphere morphogenesis of Arthrobacter crystallopoietes is inhibited by the introduction of cAMP to the culture (Ensign and Rittenberg, 1963; Kimberlin-Hariri et al., 1977). Hamilton et al. (1977) observed dramatic changes in the intracellular levels of cAMP prior to the morphological transitions of this organism, and suggested that cAMP is a contributing factor in the regulatory phenomena associated with morphogenesis. A model involving highly phosphorylated nucleotides (not cyclic) in the initiation of sporulation in B. subtilis has been presented by Rhaese and Groscurth (1976) based on the finding that sporulation in this organism can be induced by the same nutrient deficiencies that also stimulate the synthesis of highly phosphorylated nucleotides. In Caulobacter crescentus inhibition of growth and differentiation can be overcome by the addition of exogenous dibutyryl cAMP to the growth medium (Shapiro et al., 1972; Kurn et al., 1977). Schmidt and Samuelson (1972) suggested that control of stalk formation in C. crescentus appeared to be related to intracellular concentrations of nucleotides with cyclic guanosine mono-phosphate (cGMP) as a prominent candidate for an important regulatory role in this aspect of morphogenesis. Stimulation of fruiting body formation in Myxococcus xanthus was observed in the presence of exogenously supplied cAMP by Campos and Zusman (1975). Tsuboi and Yanagishima (1973) have noticed fluctuations in cAMP levels during the cell cycle of Saccharomyces cerevisiae. The addition of dibutyryl cAMP has been found to have a profound effect on morphogenesis in Mucorales (Jones and Bu'lock, 1977). During the aggregation of Dictyostelium discoideum the amoebae move towards each other in response to a chemical attractant which is believed to be cAMP (Konijn et al., 1968; Barkley, 1969; Roos et al., 1975; Newell, 1975). The involvement of cAMP oscillations as a possible control for the rate and initiation of development in D. discoideum has also been suggested by Nestle and Sussman (1972), Alcantara and Brazil (1976) and Perekalin (1977).

This introduction has served to outline a few of the major model systems available to study differentiation and morphogenesis, and to review how cellular expression may be controlled. This study was initiated to investigate differentiation and morphogenesis in a new strain of Rhodomicrobium vannielii , designated Rm 5,which was isolated and described by Dow (1974).

(vi) RhodomicrobiumRm 5 (Whittenbury and Dow, 1977). A useful model for studying morphogenesis and differentiation

Rhodomicrobium Rm 5, the isolate used throughout this study, is very closely related to Rhodomicrobium vannielii (van Niel, 1944) with regard to physiological and cultural characteristics. Addition of growth factors is unnecessary and growth occurs aerobically in the dark or anaerobically in the light (photosynthetically). Cell doubling times of approximately 4 - 5 hr are readily observed. The cells of Rm 5 are ovoid and, like R. acidophila and R. palustris possess complex internal membrane systems. Vegetative reproduction is by budding, and the sequence of events occurring during development is best described starting from a swarm cell (Fig. 1.16). The first morphologically observable event is the synchronous shedding of the flagella, producing a non-motile non-appendaged cell. This is followed after a well defined maturation period by filament formation from one or both poles of the cell, with subsequent bud formation at the filament tip. On maturation of the daughter cell a 'plug' is synthesised within the filament, physio- logically separating mother and daughter cell. Plug formation appears to be under daughter cell control, since it is always formed a finite distance from the daughter; in contrast, the distance of the plug from the mother cell is variable. Filament branching enables the mother cell to give rise to up to 4 daughter cells which usually remain attached but are separated physiologically by the plugs. Maturation of the daughter cells into mother cells, and their subsequent reproduction, results in the formation of a complex matrix of cells known as a multicellular array or microcolony.

In young cultures growing in liquid medium, motile peritrichously flagellated swarm cells are formed profusely. These cells arise from terminally attached cells and are released by binary fission (Fig. 1.17). The swarm cells are able to swim away from the microcolony, differentiate into mother cells and subsequently give rise to new microcolonies.

LEGEND

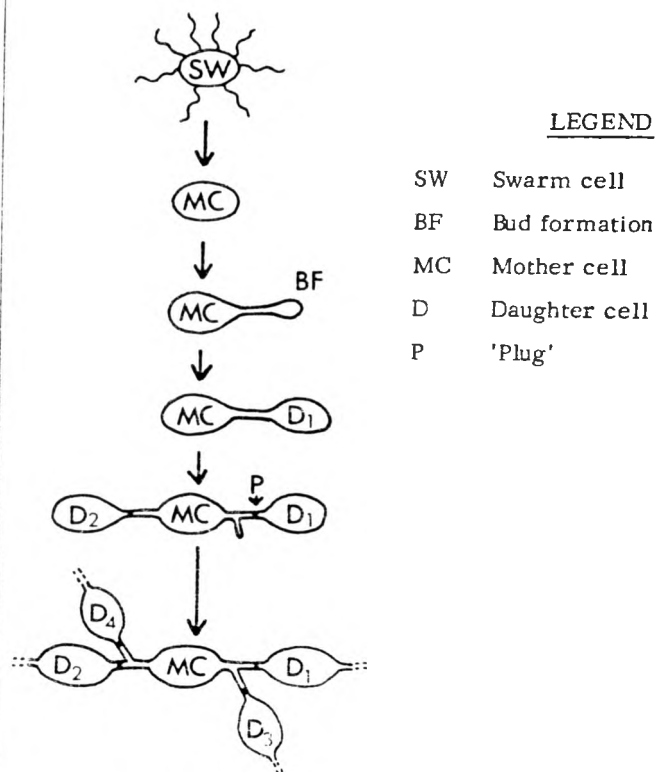|	
SW	Swarm cell
BF	Bud formation
MC	Mother cell
D	Daughter cell
P	'Plug'

Fig. 1.16

Diagrammatic representation of the vegetative growth and development of a <u>Rm 5</u> swarm cell illustrating its capacity to give rise to four daughter cells.
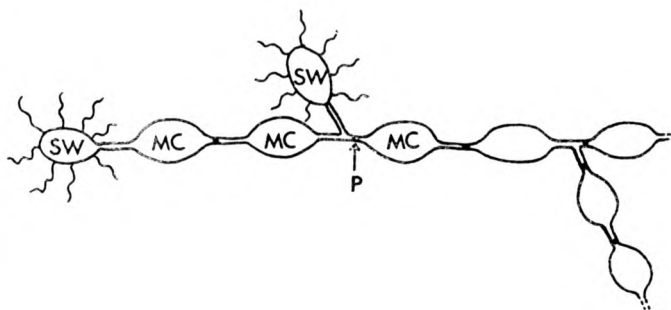
Fig. 1.17

Schematic representation of swarmer sibling formation in a <u>Rm 5</u> multi-cellular array (microcolony).

Grown under low light intensity, the culture becomes light limited before the nutrients are exhaused, all motility is lost, and resting cells or exospores are formed in abundance. These cells have a characteristic angular appearance (3, 4 or 5 sided being most common) and vary in size. Electron micrographs have shown that the spores are formed terminally on the filaments of vegetative cells and that up to 4 can be formed seuqentially from one tip (Fig. 1.18). Unlike the one to one relationship found in endospore formers, here each mother cell has the capacity to form 4 exospores, which suggests that as well as enabling the cells to survive adverse conditions, sporulation in Rm 5 also has reproductive significance. Transfer of the spores to fresh medium results in their germination and outgrowth; loss of refractility and resistant properties and the development of permeability to dyes occurs within the first hour. Vegetative cell synthesis from the exospore then follows by the character-istic budding mode of growth being preceded by filament formation from one of the apices of the angular cell (Fig. 1.19). Most spores usually generate three filaments consecutively, though occasionally 4 are observed. Exospores are unique, in so far as each is able to differentiate and become a reproductive unit capable of forming up to 4 vegetative cells in a distinctive morphological sequence. Vegetative cells formed by the exo-spore are indistinguishable from exponential vegetative cells, and upon maturation separate themselves from the exospore by plugs.

The initial work performed on Rm 5 suggests that it offers great potential as a model system for studying morphogenesis and differentiation. Features such as polar growth, polarization of the cell and mother/daughter cell cycles, involve the regulation of spatial sequences of this organism in such studies. Necessary criteria are obeyed admirably:- Rm 5 has an interesting and particularly well defined life cycle which lends itself for the correlation of biochemical events with morphological development (Whittenbury and Dow, 1977). Ease of growth in the absence of growth factors is facilitated using medium with sodium pyruvate and sodium hydrogen malate as carbon sources, and the isolation of mutants would appear to present few problems. However, a major feature of Rm 5 is the
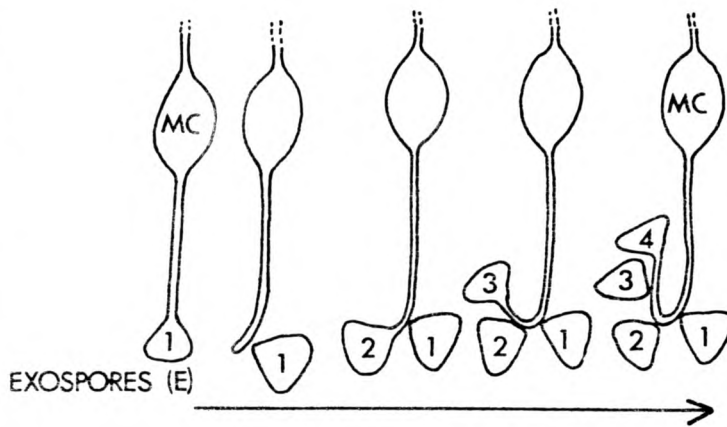
Fig. 1.18

Diagrammatic representation of exospore formation in Rm 5
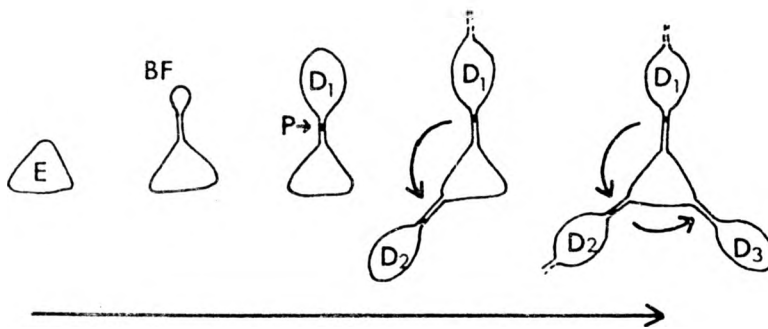
Fig. 1.19

Diagrammatic representation of exospore outgrowth

LEGEND	MC	Mother cell	D	Daughter cell
	BF	Bud formation	P	'Plug'
	E	Exospore		

ability to easily and quickly selectively synchronize the swarm cells by
filtration, with a minimum of environmental stress. The glass wool
column technique described by Dow (1974) enables vast amounts of
culture (up to 20 litres) to be synchronized, yielding in the region of
2.5×10^7 viable synchronized swarm cells per ml. Filtration as a means
of synchronization has been previously employed in B. subtilis (Sargent,
1973) and Caulobacter (Schmidt and Stanier, 1966), and the technique
has many advantages (i.e. speed, simplicity) over others currently
used for synchronizing cells, such as centrifugation and density gradient
centrifugation [E. coli (Mitchison and Vincent, 1965), R. palustris
(Westmacott and Primrose, 1976), Hyphomicrobium vulgaris (Moore and
Hirsch, 1973), Caulobacter crescentus (Stove and Stanier, 1962)] and
the Helmstetter technique and adhesion of mother cells [E. coli,
(Helmstetter and Cummings,1963, 1964:Caulobacter (Degnen and Newton,
1972a:Staley and Jordan, 1973; Kumari and Dow, personal communication)].

The following study was initiated to use Rm 5 as a model system to
investigate the morphological ultrastructural and physiological changes
occurring during the cell cycle. In particular, it was hoped to observe
whether cellular expression of this organism was influenced by the
environment and, if so, to what extent.

SECTION 2

MATERIALS AND METHODS

(i) Organisms

A strain of Rhodomicrobium vannielii isolated from freshwater by by Dr. C.S. Dow (University of Warwick) was used in all studies. This organism was designated strain Rm 5.

(ii) Gases

Carbon dioxide was obtained from the Distillers Company Limited and all other gases were from the British Oxygen Company.

(iii) Media

A basal medium containing (g/l) NH_4Cl, 0.5; $MgSO_4 7H_2O$, 0.4; $CaCl_2 2H_2O$, 0.05; NaCl, 0.4; was used for all cultures of Rm 5. This was supplemented with sodium hydrogen malate, sodium pyruvate and occasionally sodium acetate as carbon sources. Unless specified differently, growth medium contained the following carbon concentrations (g/l): -

PYR growth medium; Basal medium + sodium pyruvate (1.5)

MAL growth medium; Basal medium + sodium hydrogen malate (1.5)

PYR/MAL growth medium; Basal medium + sodium pyruvate (1.0)

+ sodium hydrogen malate (1.5)

ACET growth medium; Basal medium + sodium acetate (1.5)

Pfennig's trace element solution (Pfennig, 1969) was used where indicated (0.5 ml/litre). The pH of the medium was adjusted to 6.8 with KOH prior to autoclaving. After autoclaving sterile 0.1 M phosphate buffer, pH 6.8, (1 litre 0.1 $\underline{M}$ $Na_2HPO_4 12H_2O$ + 1 litre 0.1 $\underline{M}$ $NaH_2PO_4 2H_2O$) was added aseptically to the medium (50 ml/litre).

When media of different pH's were required, phosphate buffers were prepared from mixtures of 0.1 M $Na_2HPO_4 12H_2O$ and 0.1 M $NaH_2PO_4 2H_2O$ in proportions which gave the desired pH.

When solidified medium was required, 15 g/l of Bacto Agar (Difco Labs., West Molsey, Surrey) was added to the liquid prior to autoclaving.

(iv) Cultivation of organisms

Rm 5 was grown in a variety of ways, both in liquid culture and on solid media.

(a)   Liquid culture

Aerobically (dark)   For this purpose 250 ml conical flasks plugged with cotton wool were used.   The flasks were wrapped in aluminium foil and incubated on a rotary shaker at 30° C.

Anaerobically (light)   Here 250 ml conical flasks fitted with B19 ground glass necks and stoppered with No.37 standard turn-over type Suba-Seals (William Freeman and Co. Ltd., Staincross, Barnsley, Yorks) were most frequently used as culture vessels, although stoppered 20 ml universal bottles and stoppered 500 ml bottles were also employed.   The required atmosphere in a culture vessel was obtained by gassing with oxygen-free nitrogen via syringe needles through the Suba-Seals.   Flushing was continued until the oxygen content approached zero, as determined chromatographically.   Incubation of flasks (generally at 30° C) was either on a rotary shaker or in a reciprocating water bath.   Illumination was provided by tungsten lamps and any excess heat was dispersed by an electric fan.   Small scale liquid cultures for optical density (absorbance) measurements were prepared by completely filling and tightly stoppering 1 cm round topped glass cuvettes.   Between readings the cuvettes were incubated horizontally in a water bath or on a rotary shaker and illuminated from above.

In certain cases more specialised apparatus was required to cultivate Rm 5   and this will be described where relevant.

(b)   Solid media

Aerobically (dark)   Cultures were inoculated on to agar plates which were then wrapped with aluminium foil and incubated at 30° C.

Anaerobically (light)   Here modified 'Anaerobic Bags' were used (Westmacott and Primrose, 1975).   Inoculated agar plates were stacked up to 3 deep, lids uppermost, and taped together.   An empty dish was placed on top of the stack to minimise condensation.   Up to 6 stacks were placed on a 27 cm x 30 cm plastic tray which was covered with aluminium foil.   This tray and a 100 ml beaker containing 20 ml of saturated pyrogallol solution were then placed inside a 30 cm x 46 cm nylon bag of 0.05 mm thickness (Portex Ltd., Hythe, Kent).   Sealing of the bag was implemented using a

'Calor Easyseal' (Transatlantic Plastics Ltd.). The sealed bag was then flushed with oxygen free nitrogen via a syringe needle inserted through the nylon. A second needle served as gas outlet. Flushing was continued until the oxygen content of the bag approached zero (as determined chromatographically). 20 ml of alkali (10%(w/v)NaOH + 15%(w/v)K_2CO_3) was injected by syringe through the top of the bag into the pyrogallol. All needle holes were then sealed with adhesive tape and the completed 'bags' incubated at 30° C and illuminated by tungsten lamps (Plate 2.1).

(v) Maintenance of cultures

Stock cultures were maintained in stab cultures of PYR/MAL medium in $\frac{1}{4}$ oz vials, incubated at 30° C for five days, and stored in the light at room temperature thereafter. Duplicate stab cultures were stored at -20° C. Cultures were transferred once every six months.

(vi) Culture purity

The purity of stock strains and batch and chemostat liquid cultures was ascertained by streaking on plates of nutrient agar which were then incubated aerobically in the light at 30° C. Growth indicated the presence of contaminating organisms. Cultures were also regularly examined by phase contrast microscopy for the presence of contaminants which might not have grown on nutrient agar.

(vii) Gas concentrations in culture atmospheres

Analyses of culture atmospheres for oxygen, nitrogen and carbon dioxide were performed with a Pye-Unicam Series 104 gas chromatograph fitted with a katherometer (thermal conductivity) detector. Two 1.52 metre columns (4 mm internal diameter) were used, one filled with 'Molecular Sieve 5A' (80-100 mesh) and the other with 'Poropak R' (both obtained from Phase Separations Ltd.). The carrier gas employed was helium at a flow rate of 20 ml/min. The sytem was operated at a temperature of 50° C with a detector bridge current of 240 mA. A 0.5 ml gas sample was passed through each column and the resultant peaks recorded on a Smiths Industries Ltd. Servoscribe recorder, fitted with an integration device. 'Poropak R' resolved the sample into air and carbon dioxide
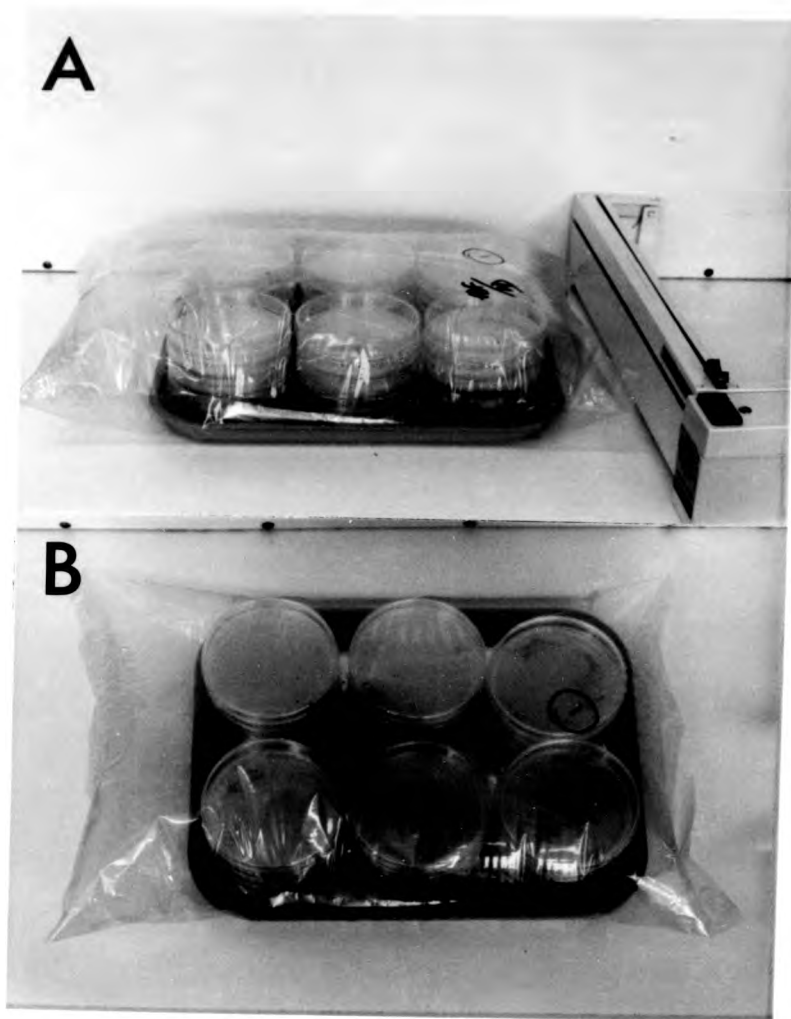
Plate 2.1

Modified 'anaerobic bags' (from Westmacott and Primrose, 1975).

Plate 2.1

Modified 'anaerobic bags' (from Westmacott and Primrose, 1975).

respectively, whereas the 'Molecular Sieve' separated the air into
oxygen and nitrogen. The minimum detection limits of separated gases
were of the same magnitude as those described by Herbert and Holding
(1972).

(viii)   Light microscopy

All cultures were examined by phase contrast microscopy. Initially
work was performed using a 'Leitz Orthoplan' microscope fitted with an
'Orthomat' camera unit, but later, however, most of the work involved
an 'Olympus E.H.T.' microscope fitted with a 'PM 6' camera unit.

Bacterial colonies on solid media were examined with an 'Olympus
Model X-Tr' stereoscopic microscope fitted with a 'PM 6' camera unit.

Photomicrographs   All photomicrographs (unless otherwise stated)
were taken on Kodak Panatomic X film (A.S.A.32). Films were developed
in Kodak D19 developer at 20° C for 3 minutes and fixed in Ilford Hypam
rapid fixer.

Slide cultures   Various methods for culturing micro-organisms
under the light microscope have been reviewed by Quesnel (1969). However,
since most of these methods were complex and needed skilful manipulation
of apparatus and materials, a simple technique was devised to enable
anaerobic growth of R.m 5 to be followed under the microscope.

Slides and coverslips were cleaned by immersion in chromic acid for
24 h   , washed with distilled water, and sterilized in absolute ethanol.
A small drop of molten agar medium was put on to a slide and a coverslip
applied immediately. Care was taken to ensure the exclusion of air
bubbles. After several minutes the coverslip was gently removed using a
fine pair of forceps, leaving a thin unbroken film of agar. A small drop
of liquid culture, sufficient to give surface cover only, was then inoculated
on to the agar and a sterile coverslip applied. Any protruding agar was
removed with a razor blade and the system sealed with a Paraffin Wax:
Vaseline mixture (1:1 w/w).

(ix)   Electron microscopy

All preparations were observed in an A.E.I. 'Corinth 275' transmission
electron microscope with a 50μ aperture and an accelerating voltage of 60 kV.

Electron micrographs were taken on 70 mm Ilford line film, N4E50, which was developed in Ilford Phenisol and fixed in Kodafix.

Shadowing   Drops of sample concentrate were placed on Parlodion - coated, 300 mesh, copper grids, fixed in OsO_4 vapour for 5 minutes and excess moisture absorbed by filter paper. Once dry, the grids were shadowed in an AE1[EC9]coating unit with gold-palladium at angles varying between 15° and 45°.

Negative staining   A similar procedure to shadowing was followed. Drops of sample concentrate were placed on Parlodion -coated, 300 mesh , copper grids, fixed in OsO_4 and excess moisture removed with filter paper. A drop of 0.5% (w/v) uranyl acetate (pH 4.5) was added and immediately removed with filter paper.

Sectioning   The fixation procedure of Ryter and Kellenberger (1958) was followed for all thin sections. Agar blocks were prepared and dehydrated with ethanol as follows:-

30% (v/v)ethanol	15 minutes
50% (v/v)methanol	15 minutes
70% (v/v)ethanol	30 minutes
90% (v/v)ethanol	30 minutes
100% (v/v)ethanol	60 minutes (2 changes).

Dehydrated blocks were then transferred to propylene oxide and then embedded in Araldite (Araldite resin CY212, 49 g; Araldite hardener D.D.S.A., 4.9 g; di-butyl phthallate (DBT) plasticiser, 0.075 g; benzyl dimethylamine (BDMA) accelerator, 0.175 g).   Blocks were allowed to polymerise at 60° C for at least 2 days.

Sections (silver) were then cut with a diamond knife on a Reichert 'OM 12' ultramicrotome.

All sections were post stained for 20 minutes in 0.5% (w/v) uranyl acetate, followed by 5 minutes in 0.1% (w/v) lead citrate in 0.1 $\underline{M}$ NaOH.

(x)   Spectrophotometry

All spectrophotometry was performed with a Pye-Unicam SP500, using 1 cm light path cuvettes. The optical densities (absorbance) of liquid cultures and suspensions were measured, with dilution where necessary, at a wavelength of 540nm (protein).   Bacteriochlorophyll was measured at a wavelength of 890 nm. For the determination of in vivo absorption spectra, cells were resuspended in saturated sucrose.

(xi)   Dry weights

Dry weights of liquid cultures were measured by filtration of a
suitable volume of culture through previously dried and weighed membrane
filters (0.4 μm). These were dried under similar conditions to the fresh
membranes in a 60° C oven and then weighed. For the sake of accuracy
the time taken between the removal of membranes from the oven and
weighing was kept constant.

(xii)   Swarm cell separation

Two techniques, both involving filtration, were employed to separate
swarm cells from a heterogeneous population. Motile swarm cells are
unhindered by passage through tightly packed glass wool, whereas non-
motile, appendaged mother cells and microcolonies become entrapped.

(a)   Glass wool column   The technique of Whittenbury and Dow (1977)
was employed. Separation of motile swarm cells from a heterogeneous
batch culture of Rm 5 was by filtration through a quick fit glass column
containing alternate layers of glass wool and 60 mesh glass chromatography
beads, introduced to prevent streaming down the sides of the column.
Sterilization of the column was at 160° C overnight in a dry heat oven.
The column was gassed with oxygen-free nitrogen in order to provide an
anaerobic environment for the cells and minimise physiological shock and
the culture was introduced to the column at a flow rate of 30-40 ml per
minute.   The first 50 ml was discarded and the subsequent filtrate was used
as the synchronised swarm cell preparation.

(b)   Syringe technique   As well as the separation of swarm cells (and pairs
and swarm cells) from heterogeneous cultures, this technique was also
used for a rapid but crude estimation of culture composition. A 50 ml
syringe was loosely packed with 6 g of washed glass wool, wrapped in
aluminium foil and sterilized by autoclaving at 121° C for 20 minutes.
40 ml of culture (absorbance x) was pushed through the syringe and the
final 6 ml collected (absorbance y). The value of (y/x x 100) gives an
approximate estimate of the culture composition, i.e. % swarm cells
(or % pairs and swarm cells).

(xiii)   Coulter counter analysis

   Particle counts and the frequency distribution of particle volumes
from liquid cultures was determined using a Model ZBI Coulter counter
(Coulter Electronics Ltd., Dunstable, Beds.) together with a Coulter
Channelyser C1000 and XY Recorder II.   Culture samples (10 μl-100 μl)
were diluted into known volumes of Isoton (Coulter Electronics, Ltd.)
which had previously been filtered through a 0.22 μm Millipore filter.
Particle counts were made immediately using a 20 μm or 30 μm aperture
tube.   Frequency distributions of particle volumes (Coulter profiles)
were accumulated in the channelyser over varying periods of time before
being printed out on the recorder.   All particle counts were converted
to counts/ml, and volumes to μm^3 using the formula

$$V(\mu m^3) = [\,(\text{Channel N}^o + \frac{\text{window width}}{100}) + B.C.T.\,] \times T_f$$

where T_f = threshold factor

This instrumentation was particularly useful for rapid counting and sizing
of swarm cells in a heterogeneous population as the time consuming process
of separation of swarm cells from microcolonies, using a glass wool
column, was eliminated.   Instrument settings most commonly used for
counting and displaying volume profiles were:-

Aperture 20 μm   Lower threshold 15

Matching switch 40 K   Upper threshold 50

Gain   10   Base channel threshold 15

Amplification $\frac{1}{2}$ Window width 100

Aperture current 1   Count range   4 K

                           Accumulation time 60 sec

(xiv)   Polyacrylamide gel electrophoresis

(a)   Cell harvesting and radiolabelling

   Organisms were harvested in the log phase of growth by centrifugation
at 4° C, washed twice with, and resuspended in, a small volume of 0.1 $\underline{M}$
phosphate buffer (pH 6.8).   When radiolabelling of protein was required,
the cells were pulsed with ^{35}S methionine (50-200 μg/ml) for 2-3 h   prior

to harvesting.   (L-[^{35}S] methionine was obtained from the Radiochemical
Centre, Amersham, Kent.   SJ 204, 200-300 Ci/mmole, 3-5 m Ci/ml in
aqueous solution containing 0.04% (v/v) 2-mercaptoethanol).

(b)   Cell disruption

All cell suspensions were disrupted by passage through a pre-cooled
'Aminco French Pressure Cell', (American Instrument Company, Silver
Springs, Maryland, U.S.A.) at 20,000 lb/sq.in and cell free extracts kept
at 4° C.

(c)   Preparation of soluble protein fraction (S.P.F.)

The disrupted cell samples were centrifuged at 38,000 g for 30 min
at 4° C, (M.S.E. Centrifuge 18L, 16 x 15 rotor, 18,000 r.p.m.) and
the clear supernatant (S.P.F.) retained. For storage purposes the
S.P.F's were frozen in dry ice/alcohol.

(d)   Protein determination

The protein concentration in the S.P.F's was determined by the Lowry
method (Lowry et al.. 1951). A standard curve was prepared using bovine
serum albumin.

(e)   Disc gels.   6% (w/v) acrylamide

Sample preparation To facilitate layering of samples on the gels, sucrose
solution (60% w/v) was added to give a final concentration of 6%(w/v) .
Proteins in the S.P.F. preparation were dissociated into polypeptide subunits
by the addition of a calculated amount of 10% (w/v) sodium lauryl sulphate
in 0.05 M Tris-HCl buffer, (pH 7.8) containing 0.1% (v/v) beta-mercapto-
ethanol such that the weight of sodium lauryl sulphate exceeded the total
weight of protein fivefold (Maizel, 1971).   Samples were then heated at
100° C in a boiling water bath for at least 3 min to eliminate any metastable
protein complexes.   Cooled samples were then loaded on to the gels. Best
results were obtained by loading 50 μg-100 μg of protein per gel.

Gel reagents

Buffer solution A	1 M HCl	48 ml
pH 8.9	Tris(trishydroxymethyl)amino-methane	36.3 g
	Sodium lauryl sulphate	0.24 g
	Water	100 ml

Acrylamide solution B	Acrylamide	24 g
	bis Acrylamide	0.48 g
	Water	100 ml

This solution was filtered after preparation and stored in the dark at 4° C.

    Initiator   Freshly prepared 0.14% (w/v)

                   ammonium persulphate in distilled

                   water.

    T.E.M.E.D.   N,N,N',N', tetramethylethylenediamine

Gel mixture The gel mixture was prepared in small ice cooled flasks and comprised:-

    Buffer solution A   5 ml

    Acrylamide solution B 10 ml

    T.E.M.E.D.   0.023 ml

    Water   5 ml

    Initiator   20 ml

Gel preparation and electrophoresis

  Gels were cast in perspex tubes, 10 cm long, 0.6 cm internal diameter, sealed at the bottom with parafilm and rubber grommets. Each tube was filled to within 1 cm of the top giving a gel rod 9 cm long. The gel mix was then carefully overlaid with a little cold distilled water to give a flat gel surface. Gels were then left to polymerise at room temperature until a sharp interface was apparent between the gel surface and the overlay. The parafilm seal was then removed and the gel tube inserted vertically into a gel tank constructed of perspex sheet (Workshop, University of Warwick). The gel tank assembly consisted of an upper tank fitting on top of a lower tank. The upper tank had grommet holes in the flow to accept the gel tubes. Both upper and lower tanks were then filled with electrophoresis buffer (pH 8.3) completely immersing the gels. Electrophoresis buffer contained per litre:-

    Glycine   28.3 g

    Tris   6.0 g

    β-mercapto ethanol 0.75 ml

    Sodium lauryl sulphate 1.0 g

Care was taken to eliminate air bubbles from the upper and lower gel surfaces.

The gel tank assembly was then connected to a Shandon Southern SAE 2761 power pack with the anode connected to the lower tank and the cathode to the top tank and the gels pre-run at 4° C, (in a cold room) using a constant current of 70 mA. The pre-run was complete when the buffer front (appears as interface) reached the bottom of the gels, and the gels were then ready for sample loading. Samples were loaded directly on to the gel tops through the electrode buffer using an Eppendorf pipette. 5 µl of tracking dye (0.1%(w/v) bromophenol blue + 6%(w/v) sucrose in a 1 in 20 dilution of electrode buffer) was also loaded on to each gel.

Running of the gels was as follows. Initially a current of 0.5 mA per gel was applied for 30 min to allow the proteins to enter the gel, and then this was increased to 2.5 mA per gel until the tracker dye reached the bottom of the gels.

Removal of the gels from the tubes was performed using a 10 ml plastic syringe filled with water. The syringe needle was inserted through a small rubber bung designed to fit securely into the top of the gel tubes and the gels extruded by applying positive pressure to the syringe.

Gel staining and destaining

Gels were allowed to stain overnight at room temperature in freshly prepared stain containing (per litre):-

Coomassie brilliant blue R250	1 g
50% (v/v) aqueous methanol	925 ml
Glacial acetic acid	75 ml

They were then destained using several changes of freshly prepared destaining solution containing (per litre):-

50% (v/v) aqueous methanol	925 ml
Glacial acetic acid	75 ml

Recording of gel patterns.

This was done in two ways.

(i) Densitometry The gel protein patterns were scanned using a Gilford spectrophotometer (Gilford Instrument Labs., Ohio, U.S.A.). The peak heights could be used to estimate protein concentration since the stain obeys 'Beers Law' over the range 0.5 - 20 µg protein (Smith, 1968).

(ii)   Photography   Gels were photographed from above by immersing in destaining solution in a transparent plastic petri dish placed on an X-ray illuminator (Industrex X-ray illuminator, model 2, Kodak Ltd., London). A Pentax SP 500 camera was used and Kodak Panatomic X (32 A.S.A.) film.

(f)   Gradient slab gels (exponential) 10% (w/v)-30% (w/v) acryalmide

This technique employed a discontinuous gel system, (Laemmli, 1970) which consisted of a stacking system with sodium lauryl sulphate added (Ornstein, 1964; Davis, 1964).

Sample preparation   Soluble protein fraction (S.P.F.) samples were prepared as previously described.

Gel reagents

High bis-Acrylamide stock:-	Acrylamide 60 g
	bis-Acrylamide 1.6 g } 100 ml
	Water
Low bis-Acryalamide stock :-	Acrylamide 60 g
	bis-Acrylamide 0.3 g } 100 ml
	Water
Stacking gel Acrylamide:-	Acrylamide 10 g
	bis-Acrylamide 0.5 g } 100 ml
	Water
Lower gel buffer:- (pH 8.8)	Tris 36.6 g
	HCl (conc.) 4.13 ml } 100 ml
	Water
Stacking gel buffer:- (pH 6.8)	Tris 5.98 g
	HCl (conc.) 4.13 ml } 100 ml
	Water
Running buffer:-	Stock 200 ml
	10% (w/v) sodium lauryl sulphate 10 ml
	Water 790 ml

(where stock buffer = 30.2 g /litre Tris + 144 g /litre glycine).

Initiator:-   Freshly prepared 10% (w/v) ammonium persulphate.

T.E.M.E.D.:-   N,N,N',N', tetramethylethylenediame.

Gel mixtures

10% (w/v) gel mix   Prepared in 100 ml conical flask:-

High bis-Acrylamide stock	8.3 ml
Water	34.9 ml
Lower gel buffer	6.25 ml
10% Sodium lauryl sulphate (w/v)	0.5 ml
T.E.M.E.D.	10 μl

30% (w/v) gel mix   Prepared in scintillation vial:-

Low bis-Acrylamide stock	10.0 ml
Glycerol 75% (w/v)	7.3 ml
Lower gel buffer	2.5 ml
10% sodium lauryl sulphate (w/v)	0.2 ml
T.E.M.E.D.	4 μl

Both gel mixes were swirled and degassed. Initiator was then added (40 μl to 30% (w/v) gel mix and 100 μl to 10% (w/v) gel mix).

Gel preparation and electrophoresis

Apparatus similar to that used by Studier (1973) was employed. The gel slab was made between two plates of window glass, one a rectangle 25 cm x 20 cm, and the other the same size, but with a notch 1.5 cm deep and 15 cm long cut from one of the 20 cm edges (Fig. 2.1). The two plates were placed together with the notch at the top and 12 mm wide (1.5 mm thick) perspex spacers were placed between the plates at the sides and bottom edges. To ensure a good seal all spacers were liberally coated with vaseline. The empty sandwich was then clamped together with bulldog clips. The gradient gel mix was then made and poured between the plates (Fig. 2.2). 10% (w/v) gel mix was pumped across into the 30% (w/v) gel mix in the mixing chamber (scintillation vial) and the bung was placed over the air outlet. The resulting pressure build up forced the gradient gel solution across into the sandwich apparatus. 50 ml of solution was pumped through and into the sandwich apparatus until the level was 3 cm below the notch. The gel solution was then carefully overlaid with water. After the gel had set ($1\frac{1}{2}$-$2\frac{1}{2}$ h) the overlaying water was removed and the degassed stacking gel mix was added using a syringe. This comprised:-

Fig. 2.1

Gradient slab gel 'sandwich' plates
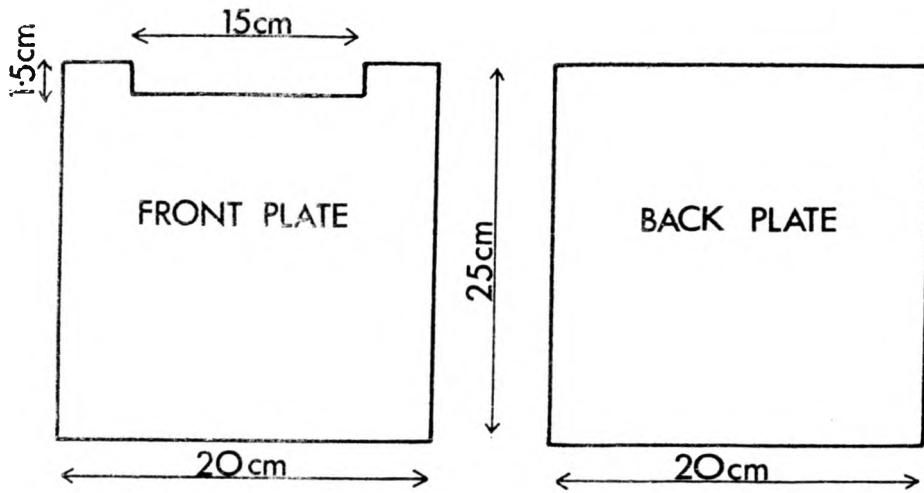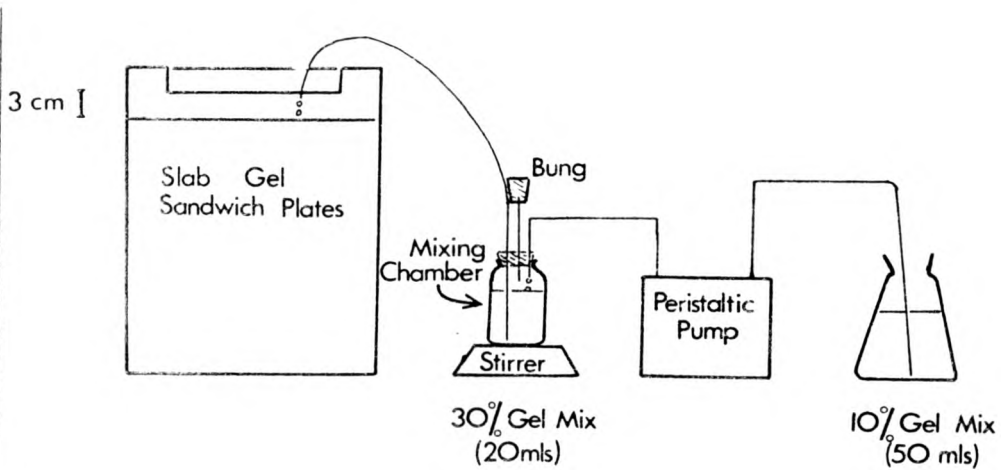

Fig. 2.2

Gradient slab gel apparatus

10% (w/v) stacking gel Acrylamide	3.0 ml
Water	4.4 ml
Stacking gel buffer	2.4 ml
Sodium lauryl sulphate 10% (w/v)	0.1 ml
T.E.M.E.D.	5 μl
Ammonium persulphate 10% (w/v)	0.1 ml

A perspex slot former was immediately pushed into the stacking gel which was then allowed to set ($\frac{1}{2}$-1 h). The slot former was then carefully removed and the sample wells filled with running buffer. All bulldog clips and the bottom perspex spacer were removed from the plates which were then fastened to the electrophoresis apparatus (Workshops, University of Warwick). The front face of the sandwich was liberally coated with vaseline and the sandwich clamped to the apparatus so that the notch in the glass plate was next to a similar notch in one side of the upper buffer chamber. The purpose of the vaseline was to form a liquid tight seal and prevent leakage of running buffer from the top reservoir. Both upper and lower electrophoresis tanks were filled with running buffer and the S.P.F. samples and molecular weight standards loaded directly into the sample wells using an Eppendorf pipette (approximately 20 μg -100 μg protein in 50 μl per well).

Any bubbles on the bottom of the gel were removed by a stream of running buffer from a cannula which was bent at the end and attached to a 10 ml syringe. 5 μl of 0.1% (w/v) bromophenol blue tracker dye was added to each of the sample wells and the gels were run at 20 mA constant current until the dye front reached the bottom of the gel. A Shandon Southern SAE 2761 power pack was used with the anode connected to the lower reservoir and the cathode to the upper reservoir. After completion of electrophoresis the sandwich plates were removed from the electrophoresis apparatus and the two remaining spacers removed. The sandwich plates were then prised apart and the gel slab carefully transferred to a large sandwich box for staining.

Gel staining and destaining   Gels were completely submersed in freshly prepared stain (45% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie blue) for a minimum of 3 h . The sandwich boxes were gently agitated on a rotary shaker.

Destaining followed, using a set sequence:-

    Destain solution 1. (45% methanol (v/v), 10% acetic acid (v/v)) 3-4 h .
    Destain solution 2. (20% isopropanol (v/v), 10% acetic acid (v/v)) overnight.
    Destain solution 3. (10% isopropanol (v/v), 10% acetic acid (v/v)) to completion.

Recording of gel patterns

Photography   Gels were photographed from above by sandwiching between
2 glass plates which were then placed on an X-ray illuminator. A Pentax
SP 500 camera was used and Kodak Panatomic X (32 A.S.A.) film.

Gel drying and autoradiography   Gels employing radio-labelled samples
were dried down and autoradiographed. The technique of Maizel (1971)
was used for drying down the gels. The gel drying apparatus consisted of
a sheet of surgical grade silicone rubber (1/32" thick), 12 inches square,
on to which the gel was laid. A piece of Whatman 3MM filter paper was
placed on top of the gel and a piece of porous polypropylene was laid on
top of that, smooth side down. Another 12 inch square of silicone rubber,
with a 5/16 inch hole in the centre to accommodate a steel tubing connector,
was placed on top of the polypropylene. A vacuum was then applied using
a water aspirator vacuum pump, and this sealed the silicone rubber around
the gel. The complete assembly was then immersed in a water bath at
80° C and the gel allowed to dry down (1-2 h).

Dried down gels were autoradiographed using Kodirex X-ray film and
exposed for 1-2 weeks. Autoradiographs were developed using Kodak DX
80 X-ray developer and fixed in Kodak FX 40 fixer. They were photo-
graphed using an X-ray illuminator, a Pentax SP 500 camera and Kodak
Panatomic X film. Quantitation of autoradiographs was achieved by
densitometry of the developed film. Here a Chromoscan recording and
integrating densitometer was employed (Joyce Loebyl and Co. Ltd.,
Gateshead, England).

(g) Two dimensional gels

The technique used was that of O'Farrell (1975). Proteins were
separated according to isoelectric point by isoelectric focussing in the
first dimension, and according to molecular weight by sodium lauryl
sulphate (S.D.S.) electrophoresis in the second dimension.

Sample preparation   S.P.F. extracts (radiolabelled) were prepared as previously described.   However, prior to cell disruption the pellet was resuspended in lysis buffer and not standard phosphate buffer.

First dimension   Isoelectric focussing gel

Gel reagents

Lysis buffer

Ultra pure urea	5.7 g	
Nonidet P-40	0.2 g	
Ampholines (pH 3.5-10)	0.5 ml	100 ml
β-mercapto-ethanol	0.5 ml	
Water		

Acrylamide stock

Recrystallised Acrylamide	2.84 g	
Recrystallised bisAcrylamide	0.162 g	100 ml
Water		

Gel overlay solution

Ultra pure urea	48% (w/v)

Sample overlay solution

Ultra pure urea	5.4 g	
Ampholines		10 ml
Water		

Initiator   Ammonium persulphate 10% (w/v)

Electrophoresis buffer

0.01 M H_3PO_4	Anode
0.01 M NaOH	Cathode

Gel mixture

Ultra pure urea	2.75 g
Acrylamide stock	0.67 ml
10% (v/v) NP-40	1.00 ml
Water	0.99 ml
Ampholines	0.25 ml
T.E.M.E.D.	3.5 μl

Gel preparation and electrophoresis

The gel mixture was degassed under vacuum and 10 μl of initiator added.   Loading into the gel tubes (glass tubing 120 mm x 2.5 mm internal diameter sealed at the bottom with Parafilm) followed

immediately, care being taken to exclude air bubbles. The gel was
overlaid with gel overlay solution, and after a further 2 h this overlay
solution was removed and replaced with 20 μl of lysis buffer and
further overlaid with a small amount of water. When the gels had set
(2 h) the Parafilm was removed and the ends of the tubes covered with
dialysis membrane held in place by a latex grommet. The gel tubes
were then placed in a standard tube gel electrophoresis chamber and
the lysis buffer and water removed from the gel surfaces. This was
replaced with 20 μl of fresh lysis buffer and the tubes filled with 0.01 M
NaOH. Both upper and lower tanks were then filled with degassed
0.01 M NaOH and 0.01 M H_3PO_4 respectively and connected to a
Shandon Southern SAE 2761 power pack. The gels were pre-run as
follows:-

<div align="center">

200 volts for 15 min

300 volts for 30 min

400 volts for 30 min

</div>

Power was then turned off, the upper tank was emptied and the lysis
buffer and NaOH removed from the surface of the gels.

S.P.F. samples (20-40 μg/20 μl lysis buffer) were then loaded on
using an Eppendorf pipette, and overlaid with 10 μl of sample overlay
solution and 0.01 M NaOH. The tank was then refilled with electro-
phoresis buffer and the gels run overnight at 400 volts.

To remove the gels from the tubes, a 5 ml syringe was connected
to the electrophoresis tube (via Tygon tubing) and the gel was slowly
extruded by pressure on the syringe. Gels were then either loaded
immediately on to the second dimension or stored frozen.

Second dimension:-

Gel reagents. Same as for the 10% (w/v)-30% (w/v) slab gel as previously
described. In addition the following were required:-

      Agarose gel   1 g of agarose melted in 100 ml of sample
                                 buffer + 2 ml 0.1% (w/v) Bromophenol blue.

<u>Sample buffer</u>

Stacking gel buffer	1.25 ml
Glycerol	1.0 ml
10% (w/v) sodium lauryl sulphate	2.0 ml
β-mercapto-ethanol	0.5 ml
0.1% (w/v) Bromophenol blue	0.1 ml
Water	5.25 ml

<u>Gel mixture</u>   As described for the 10%(w/v)-30% (w/v) slab gel.

<u>Gel preparation, loading and electrophoresis</u>

A 10%$^{(w/v)}$-30%$^{(w/v)}$slab gel was prepared as previously described. However, since the perspex slot former was not required, the stacking gel was overlaid with distilled water. Two P.T.F.E. tabs were inserted at either end of the stacking gel and these, when removed, served as wells for molecular weight markers. Before loading, the isoelectric focussing gel (I.F.G.) was equilibriated for 2 h in sample buffer. Overlaying water was then removed and the I.F.G. loaded as follows. It was placed on a piece of Parafilm, straightened out, and arranged close and parallel to one edge. Melted agarose was then pipetted into the notch of the slab gel sandwich and the Parafilm used to quickly transfer the cylindrical gel into this solution. Molecular weight markers were loaded into the wells on either side of the I.F.G.   When the agarose had set, the slab gel was run as previously described, using 20 mA constant current.

<u>Staining, destaining, drying, autoradiography</u>

This was all performed as previously described for 10% (w/v)-30% (w/v) slab gels.   Dried down gels were autoradiographed using Kodirex X-ray film. A considerably longer exposure time (1-3 months) was required however. Autoradiographs were developed using Kodak DX 80 developer and fixed using Kodak FX 40 fixer.   They were photographed on an X-ray illuminator using a Pentax SP 500 camera and Kodak Panatomic X film.

(XV)   <u>Continuous culture</u>

During this study 3 different types of continuous culture apparatus were employed.

(a) Simple chemostat (Fig. 2.3)

Initial runs were performed using a cheap chemostat constructed of Jobling glassware. The basic fermenter unit employed a 100 ml sidearm conical flask with a working volume of 70 ml. There was no provision for pH or temperature control and all 'runs' were performed at 30° C in a warm room. Agitation was by means of a magnetic follower and illumination by a tungsten lamp. Excess heat generated by the magnetic stirrer and lamp was transmitted away from the fermenter by an electric fan. The culture was kept anaerobic using sterile filtered oxygen-free nitrogen, and culture samples were taken from the overflow using a sampling hood and sterile universal bottles. Performance of this apparatus, however, was far from satisfactory. The absence of pH control and small working volumes were large disadvantages, and backgrowth of contaminants from the sampling hood and clogging also presented problems.

(b) L.H.E. 2 litre chemostat

Two litre continuous cultures of Rm 5 were grown using a L.H.E. 1/1000 laboratory fermenter (L.H. Engineering Co. Ltd., Stoke Poges, Bucks, England) fitted with temperature and pH control. The culture was kept anaerobic using sterile filtered oxygen-free nitrogen. Light energy was supplied by means of a 100 watt tungsten lamp with loss of illumination minimised by covering the sides of the fermenter housing with aluminium foil. Incubation was at 30° C with a stirring rate of 100 rpm and pH was controlled by the addition of 0.1 M HCl.

(c) Quickfit chemostat (Plate 2.2, Fig. 2.4)

This fermenter evolved from the simple chemostat and provided a cheap and convenient system for following the behaviour of Rm 5 in continuous culture. The growth vessel was comprised of a Quickfit FV1L culture vessel and flanged lid, giving a working volume of 700 ml. Culture temperature was controlled by circulating water around the water jacket (a 2500 ml beaker) using a Churchill thermocirculator, and agitation by means of a magnetic stirrer. pH control, using gaseous CO_2 and/or 0.1 M HCl, was facilitated by means of an electrode coupled
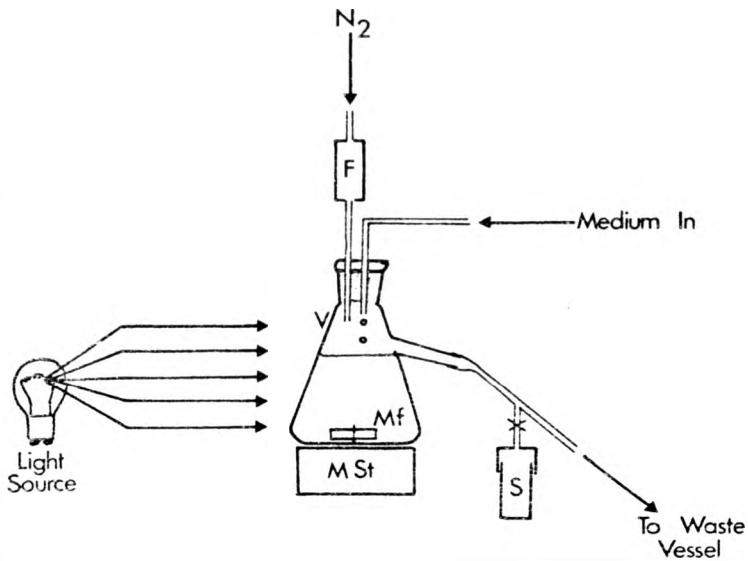
Fig. 2.3

Simple chemostat

LEGEND

F	Filter ('Gamma' 0.3 μm)
Mf	Magnetic follower
MSt	Magnetic stirrer (Gallenkamp Ltd.)
S	Sample port
V	Fermenter vessel
X	Tap

Plate 2.2

'Quickfit' chemostat and ancillary apparatus

LEGEND   (For Fig. 2.4 overleaf)

Ac	Acid reservoir
Al	Alkali reservoir
AV	Constant volume head
F	Filter (Gamma $0.3\ \mu m$)
G	Gas flow meter
L	Light source (angle poise lamp, 150 watt)
MR	Medium reservoir.
WR	Waste reservoir
MSt	Magnetic stirrer
MI	Fresh medium input
mf	Magnetic follower
P	Phosphate buffer reservoir
PP	Peristaltic pump
pH	pH controller and output to pump
S	Sample port
T	Temperature controller
X	Tap
$\rightarrow$	Direction of gas, liquid or electric impulse

Plate 2.2

'Quickfit' chemostat and ancillary apparatus

LEGEND   (For Fig. 2.4 overleaf)

Ac	Acid reservoir
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pH	pH controller and output to pump
S	Sample port
T	Temperature controller
X	Tap
$\longrightarrow$	Direction of gas, liquid or electric impulse

Fig. 2.4

Diagrammatic representation of 'Quickfit' chemostat system

to an industrial meter and controller. Anaerobiosis was maintained by a constant flow (20 ml/min) of sterile filtered oxygen free nitrogen bubbling through the culture. The pressure from the nitrogen also provided a gas lift for maintaining a constant culture volume.

The fermenter vessel was filled with medium and sterilized at 121° C for 25 min with all accessories, connecting silicone tubing and 20 litre reservoir and overflow bottles attached. Fresh medium refills were pumped into the reservoir from a previously sterilised 20 litre supply containing phosphate buffer, via 0.3 μm 'Whatman Gamma' in line filters, to exclude any possible contaminating organisms. Phosphate buffer and starter inocula were injected into the fermenter vessel through a Suba Seal in a spare port. Cell samples were withdrawn through narrow stainless steel tubing attached to a sampling hood accommodating sterile 20 ml universal bottles. Closing off the gas outlet on the fermenter vessel enabled pressure to build up and so push the culture out.

(XVI)   15 litre batch fermenter   (Plate 2.3,   Fig. 2.5)
             Culture conditions   15 litre batch cultures of/Rm 5 were grown
Rhodomicrobium
under differing conditions using a L.H.E. 15 litre fermenter (L.H. Engineering Co. Ltd., Stoke Poges, Bucks, England) fitted with temperature and pH control. Anaerobiosis was attained by flushing with sterile filtered oxygen-free nitrogen.   Light energy was provided by a 150 watt tungsten lamp positioned 6 inches from the fermenter vessel. By covering the sides of the fermenter housing with aluminium foil, loss of light and culture shading was kept to a minimum. Incubation was at $3C^o$ C with a stirring rate of 100 rpm. The pH was controlled where necessary by the addition of sterile 0.1 $\underline{M}$ HCl or 0.1 $\underline{M}$ KOH.

Sterilization of the fermenter, filled with medium, and all accessories attached, was facilitated by autoclaving at 121° C for 25 min. Phosphate buffer and starter inocula were injected into the fermenter vessel through a Suba Seal in a spare port.   Cell samples were withdrawn (by siphoning or pressure build up) through stainless steel tubing attached to a sampling hood.

Plate 2.3

(A)   15 litre batch fermenter and ancillary apparatus.
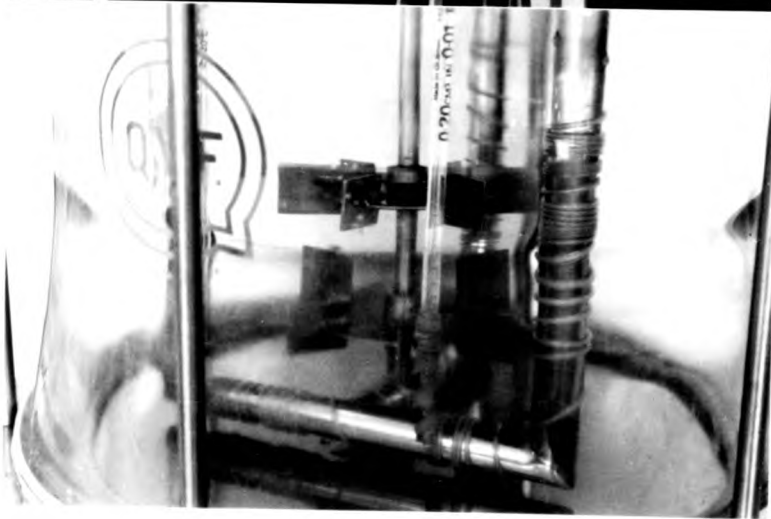
(B)   Close-up of silicone gas detection coil.

Plate 2.3

(A) 15 litre batch fermenter and ancillary apparatus.
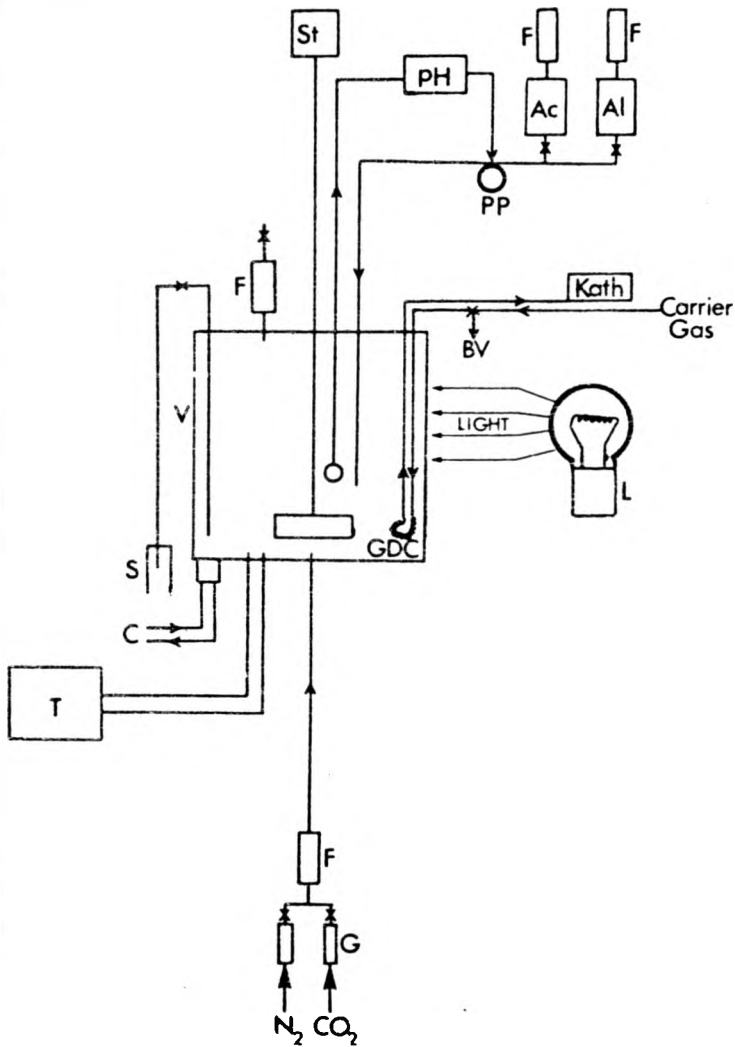
(B) Close-up of silicone gas detection coil.

Fig. 2.5

Diagrammatic representation of 15 litre batch fermenter system

LEGEND

Ac	Acid reservoir	L	Light source
Al	Alkali reservoir	PP	Peristaltic pump
BV	Bipass valve	pH	pH flow meter and output to pump
C	Cooling finger	S	Sample port
F	Filter ('Gamma' $0.3\,\mu m$)	St	Stirrer motor
G	Gas flow meter	T	Temperature controller
GDC	Gas detection coil	V	Fermenter vessel
Kath	Katherometer detector	X	Tap

Gas analysis

Detection of dissolved carbon dioxide, oxygen and nitrogen in the
culture was effected by adaptation of a method described by Philips and
Johnson (1961) and Roberts and Shepherd (1968).   Helium carrier gas
was passed at 20 ml/min through 7 metres of silicone tubing, 1 mm
internal diameter, 0.5 mm wall, (Esco Rubber Ltd., Great Portland St.,
London) which was completely submerged in the fermenter liquid and
coiled about horizontal and vertical axes.   The carrier gas was introduced
into the silicone coil from the katherometer exhaust gas outlet via 1.5 mm
internal bore teflon tubing and a glass T junction (bipass valve).   From
the silicone coil the carrier gas and sample gases were introduced into
the 0.5 ml sample loop of the katherometer by means of similar teflon
tubing and accessory couplings.   Samples were taken at regular time
intervals and to ensure reproducibility a strict routine was followed:-

(a)   Close bipass valve V and allow helium to flow through the coil.

(b)   Equilibriate for 2 minutes

(c)   Inject sample into column (Poropak R or Molecular Sieve)

(d)   Open bipass valve

The routine was repeated after 5 min using a $1\frac{1}{2}$ min equilibriation time.
The resultant gas peaks were recorded on a Smiths Industries Ltd.,
Servoscribe recorder, fitted with an integration device for calculating
the area under each peak.   Attenuations used were :-

Oxygen	5
Nitrogen	100 or 200
Carbon dioxide	100 or 200

The dissolved nitrogen and oxygen tensions remained constant for the
duration of each 'run'.   The amount of carbon dioxide present was
expressed as a percentage of the total dissolved gas atmosphere.

$$\text{i.e.} \quad \% \, CO_2 = \frac{[CO_2]}{[N_2] + [O_2] + [CO_2]} \quad \times \, 100$$

Culture sample analysis

Samples were taken at regular intervals and analysed as follows:-

(i) Absorbance 540 nm and 890 nm

(ii) pH

(iii) Swarm cell count

(a) <u>Viable</u> Swarm cells were separated using a glass wool column :-
  glass wool plug
  15 g large beads
  30 g 40 mesh heads Column
  15 g large beads
  glass wool plug

The column was washed with 50 ml of medium. 100 ml of culture was passed down and the first 25 ml of eluent discarded. The following 50 ml was collected, serially diluted, and plated out (anaerobic bag).

(b) <u>Total</u> Coulter counter

(iv) Swarm cell volume and Coulter profile. Coulter channel lyser.

(v) Dry weights. Total culture. Swarm cells.

(XVII) <u>Carbon dioxide uptake</u>

Determination of the uptake of exogenous carbon dioxide was carried out by incubating cells in the presence of ^{14}C labelled carbon dioxide (sodium [^{14}C] bicarbonate was obtained from the Radiochemical Centre, Amersham, Kent, CFA 3, 56.5 Ci/mmole, 656 μ Ci/mg in dilute sterilized aqueous solution). 1 litre aspirators, with rubber bladders providing gas atmospheres, were used as culture vessels (Plate 2. 4). Log phase cells were used to inoculate the aspirators which contained 700 ml of standard PYR/MAL medium and 0. 1 $\underline{M}$ phosphate buffer (pH 6. 8). Anaerobiosis and inflation of the bladder was effected by flushing with oxygen-free nitrogen and then 35 μ Ci of labelled carbon dioxide was added to each culture by syringe.

1 ml samples were removed at regular intervals and label incorporation stopped by dilution with 10 ml of cold 10% (w/v) T.C.A. The samples were then filtered on to 2.5 cm Whatman GF/C glass fibre filters, washed three times with 5 ml of cold 10%/T.C.A. and dried overnight in a 60° C oven.
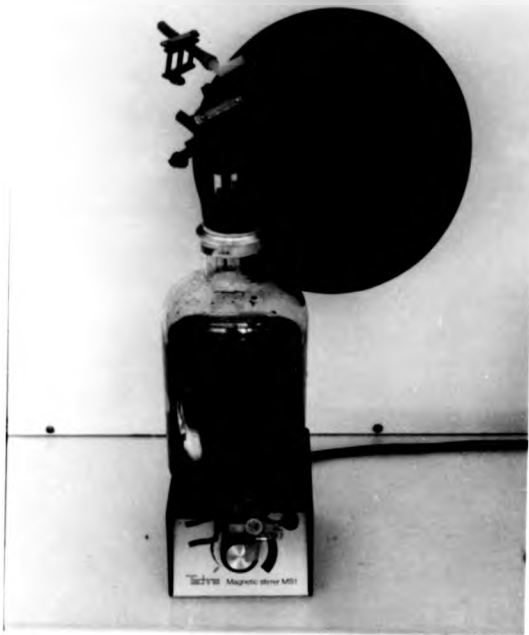
Plate 2.4

Culture vessel used for labelled CO_2 uptake measurement

Plate 2.4

Culture vessel used for labelled CO_2 uptake measurement

Glass fibre filters were counted by immersion in scintillation fluid, consisting of 36 g 2-5-diphenyloxazole plus 0.45 g 1, 4-bis-[5-phenyloxazole-2-yl]benzene dissolved in 6 litres of toluene plus 3 litres of Triton-X-100, on a Packard 3000 series Tri-Carb liquid scintillation spectrometer.

(XVIII) Heat resistance of 'minispores'

Cells from the culture (containing 'minispores') were harvested and washed thoroughly with 0.1 M phosphate buffer (pH 6.8). Exponentially growing cells from a chemostat were used as control. Both cultures were heat treated at 60° C in thin walled glass tubes which were totally immersed. 0.1ml samples were taken at intervals, serially diluted and spread on to PYR/MAL agar plates. Incubation of the plates was in anaerobic bags at 30° C, with illumination of 1000 lux.

Section 3   RESULTS AND DISCUSSION

(a)   Selection of Rm 5 cells with a simplified cell cycle ('pairs and swarms').

(b)   Some characteristics of pairs and swarm (P + S) cells.

(c)   Polyacrylamide gel electrophoresis studies.

(d)   Continuous culture studies.

(e)   Simplified cell cycle.   Mutation or a response to an environmental stimulus ?

(f)   15 litre batch fermenter studies.

(a) Selection of Rm 5 cells with a simplified cycle

The growth of Rhodomicrobium in batch culture has been well documented (Duchow and Douglas, 1949; Douglas and Wolf e, 1959; Gorlenko, 1969; Whittenbury and Dow, 1977), and is characterised by changes in morphology and functional cell expression during specific growth phases. A number of distinct cell types (Plate 3.1) can be observed:-

Stalked mother cells, which form chains of cells, which may be branched, giving rise to multicellular arrays or microcolonies.

Motile swarm cells, which are non-appendaged and peritrichously flagellated.

Exospores. Angular resting cells which are very resistant to adverse conditions.

The behaviour of such a complex organism in continuous culture has not been studied.

Simple chemostat

Fig. 3.1 illustrates the behaviour of a heterogeneous culture of Rm 5 in the simple chemostat (working volume 70 ml, temp 30° C, illumination 2500 lux). A midexponential phase inoculum of Rm 5 was allowed to grow to mid-log phase in the chemostat (absorbance$_{540}$ ~ 1.8) before continuous flow of fresh medium was initiated at the lowest possible rate (7.8 ml/h). Wash-out occurred and when the culture absorbance fell below 0.9 it was rebatched and allowed to recover. On reaching mid-log phase, continuous flow was once again initiated and wash-out re-occurred. Considerable wall growth was evident on the side of the fermenter and this was continually removed by scraping. The batch/run cycle was repeated several times, and eventually a steady state was obtained after approximately 10 days.
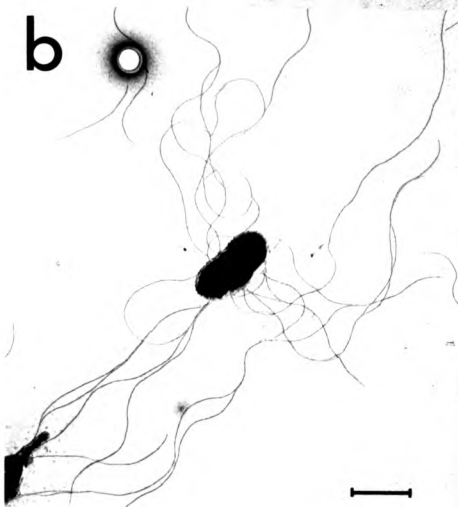
Steady state:-   absorbance$_{540}$ 1.02

absorbance$_{890}$ 0.63

pH   8.1

dilution rate   0.11 h^{-1}

In steady state there was an absence of wall growth, and microscopic examination revealed a considerable change in culture composition. Virtually all of the microcolonies or multicellular arrays had disappeared, leaving a
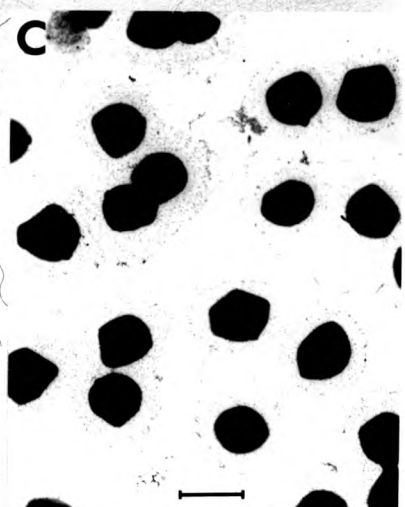
Plate 3.1

(Bars = 1 μm)
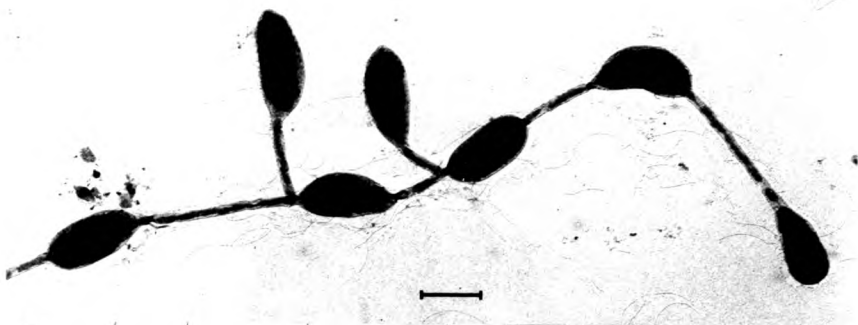
Electron micrographs (gold palladium shadows) of the distinct cell
types of Rm5

(a)   Stalked mother cells comprising a multicellular array

(b)   Peritrichously flagellated swarm cell

(c)   Angular resting cells or exospores
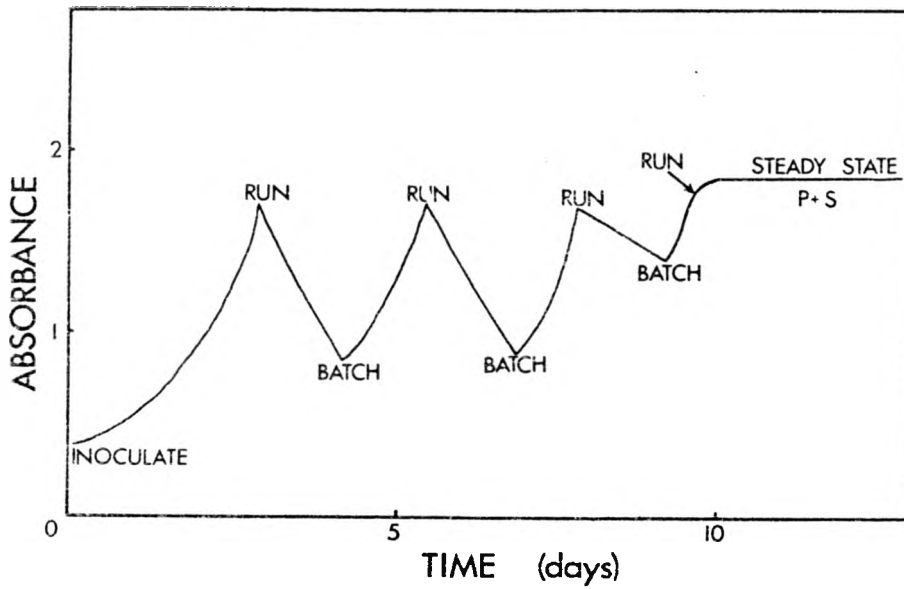
Fig. 3.1

Behaviour of a heterogeneous culture of 'normal' Rm 5 in
continuous culture. (Simple chemostat, 30° C, 2500 lux,
dilution rate $0.11h^{-1}$). Steady state obtained after repeating a
batch/run cycle.
Mutant selection ?

culture composed almost entirely of swarm cells and swarm cell-mother cell duplexes (pairs) (Plate 3.2). The dimensions of a single swarm cell was, on average, 1.5 μm x 0.8 μm, whereas mature cells in a 'pair' could be as large as 3 μm x 1 μm. Filament diameter was constant and between 0.15 μm - 0.2 μm, but filament length varied and ranged in length from 0.4 μm to 1.0 μm.

Cells from such a culture have a characteristic 'Coulter profile', comprising of a peak and a broad shoulder (Plate 3.3). This profile represents the swarm cells (mean volume ~0.82 μm^3) and the volume changes associated with the stages of development, through to the formation of a swarm cell-mother cell pair (i.e. swarm cell maturation, tube and bud formation). If pairs and swarm (P + S) cells are allowed to grow to stationary phase, most of the pairs eventually separate and a culture composed of single cells and single cells with an appendage is observed. The 'Coulter profile' changes and a drop in cell volume is also evident (Plate 3.3).

In contrast the 'Coulter profile' of a 'normal' heterogeneous culture of Rm 5 .growing exponentially,is composed of a large swarm cell peak and a very small shoulder (Plate 3.4). In such a culture, swarm cells are produced in large numbers. However, at any one time only a small percentage are developing, and the microcolonies have volumes too high to be observed on the same scale.

The isolation of 'P + S' cells would appear to be a classic example of the use of continuous culture for mutant selection. Most gene mutations are spontaneous and arise during the active growth of cells in the absence of known mutagens. The frequency of spontaneous mutants in a culture composed initially of wild type bacteria increases linearly with time when there is no selection for or against the mutants. If the growth rate exceeds that of the wild type, the rise of mutant frequency becomes accelerated as time progresses, until the culture contains a small percentage of wild types. The use of continuous culture as a tool for microbial selection has been widely reviewed (Veldkamp and Jannasch, 1972 ; Meers, 1973; Jannasch and Mateles, 1974; Harder et al., 1977). Mutants of Brucella abortus (Braun et al., 1951).
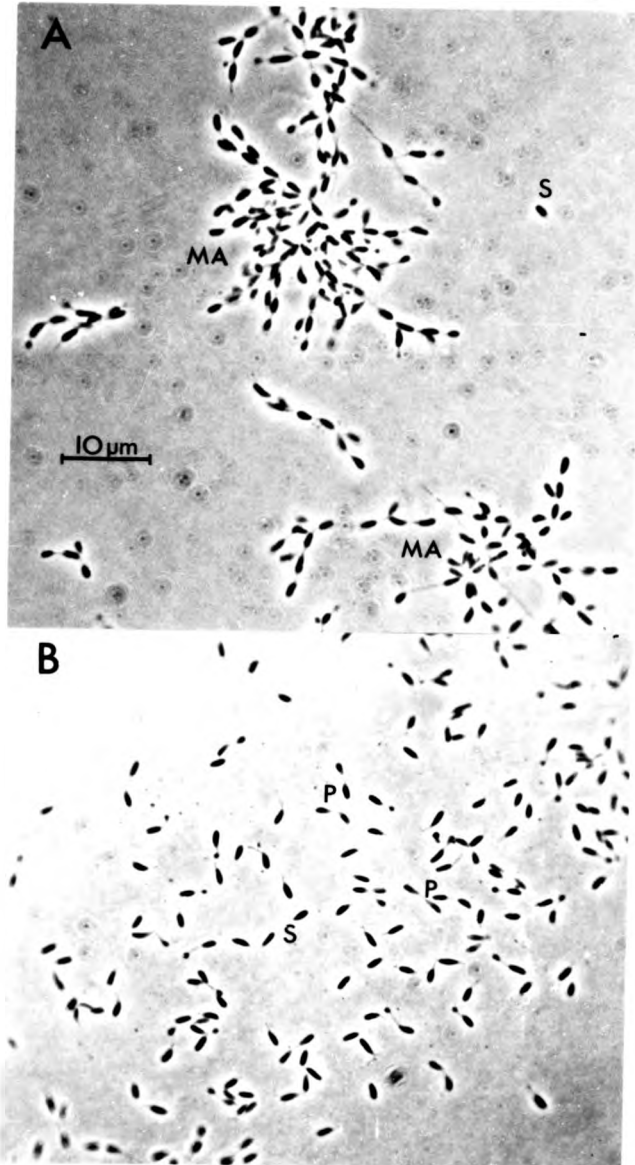
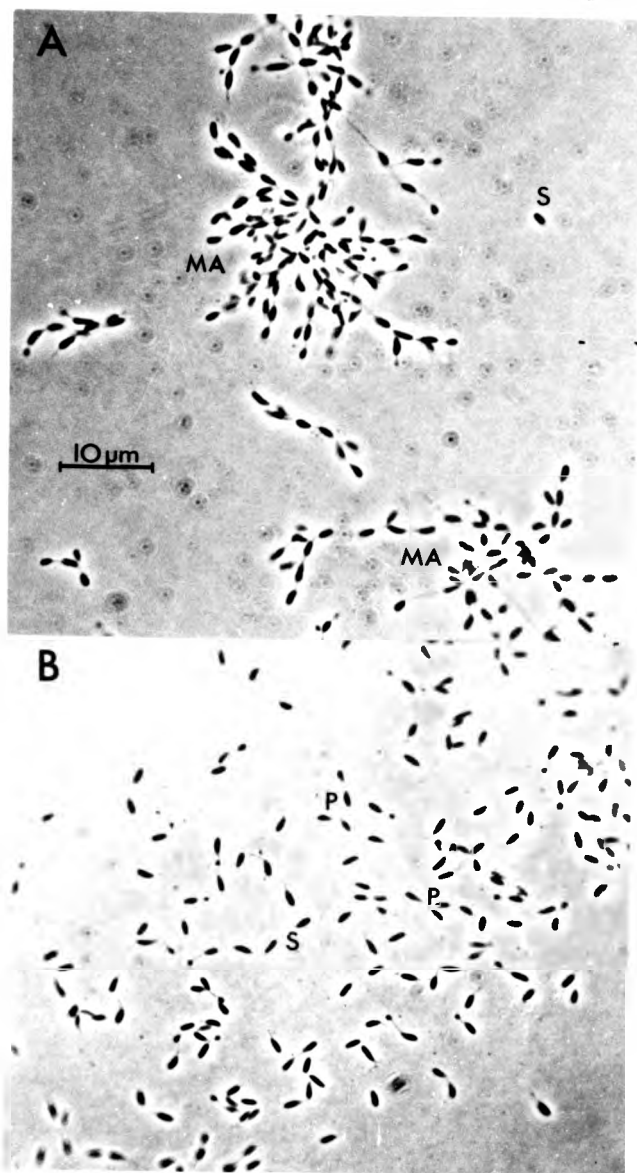Plate 3.2

Light photomicrographs   (phase contrast)

(A)   A heterogeneous population of 'normal' Rm 5 cells, comprising
       swarm cells (S) and multicellular arrays (MA) before
       continuous culture.

(B)   Cells from the chemostat steady state.   Culture composed almost
       entirely of pairs (P) and swarm cells (S).

Plate 3.2

Light photomicrographs   (phase contrast)

(A)   A heterogeneous population of 'normal' Rm 5 cells, comprising
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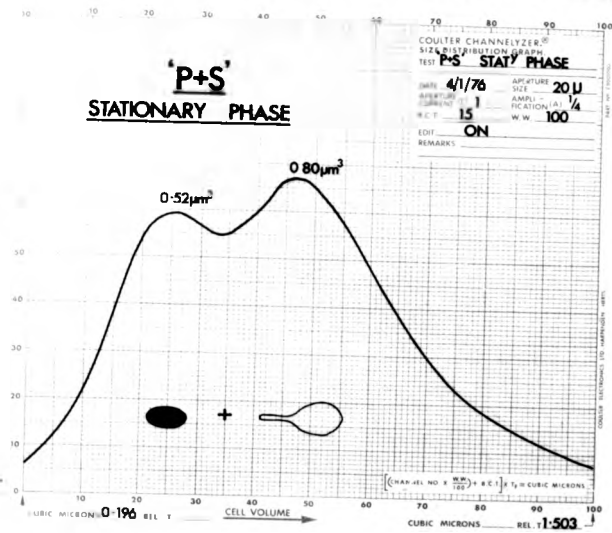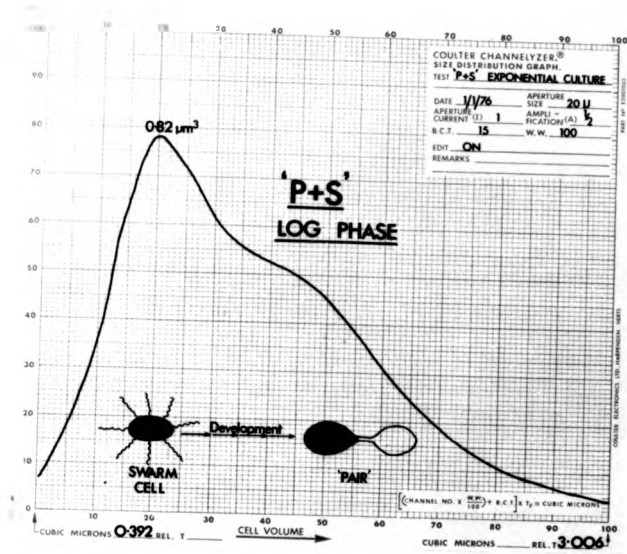
Plate 3.3

'Coulter' profiles'

(A)   Exponential growth phase P + S cells from the chemostat
        steady state.
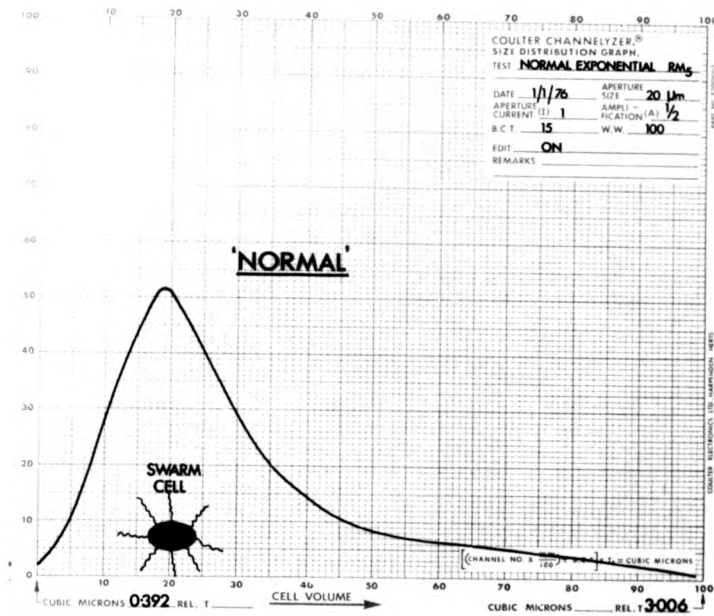
(B)   Stationary phase P + S cells.

Plate 3.4

'Coulter profile' of a 'normal' heterogeneous culture of Rm 5.
Only the swarm cell peak is evident as the microcolonies have
volumes too high to be observed on the same scale.

E. coli (Cocito and Bryson, 1958), Klebsiella aerogenes (Rigby et al., 1974) have been selected using this technique. Chemostat selection on the basis of differences in the response of Pseudomonas sp and Spirillum sp to more than one environmental factor, has been illustrated by the work of Harder and Veldkamp (1971).

Further work described later in this thesis using batch and continuous culture techniques suggests that P + S cells are not necessarily mutants, and may be produced as a response to an environmental stimulus, e.g. they can be established by the growth of Rm 5 in the presence of high CO_2 concentrations (2.5×10^4 p.p.m.).

Many of the cell pairs from the steady state chemostat were actively motile. Electron microscope studies revealed that this was due to the presence of peritrichous flagella on one of the cells making up the 'pair' (Plate 3.5).

Unlike 'normal' vegetative cells, cross wall 'plugs' separating the two cells were never observed. P + S cells obtained from the steady state conditions of the chemostat were reasonably stable. Log phase cells could be subcultured many times before substantial numbers of microcolonies and chains began to reappear. However, subculturing of stationary phase cells promoted a more rapid reappearance of multicellular arrays.

Likewise on agar plates they are stable and remain in the P + S condition. Cells from a P+S culture, however, give rise to colonies of totally different morphology from those of 'normal' vegetative cells. P + S colonies are smooth, pale and have a regular outline. They are mucoid and easily disrupted. Multicellular arrays give rise to darker, pitted, matt colonies which are irregular, tight and difficult to disrupt (Plate 3.6).

Measurement of the in vivo absorption spectrum of the P + S cells showed that the altered pigmentation of the colonies on plates was not due to a change in pigment composition. Both 'normal' heterogeneous Rm 5 and P + S Rm5 have identical absorption spectra with carotenoid peaks at 460, 492 and 526 nm, and bacteriochlorophyll a peaks at 375, 596, 807 and 875 nm (Fig. 3.2).
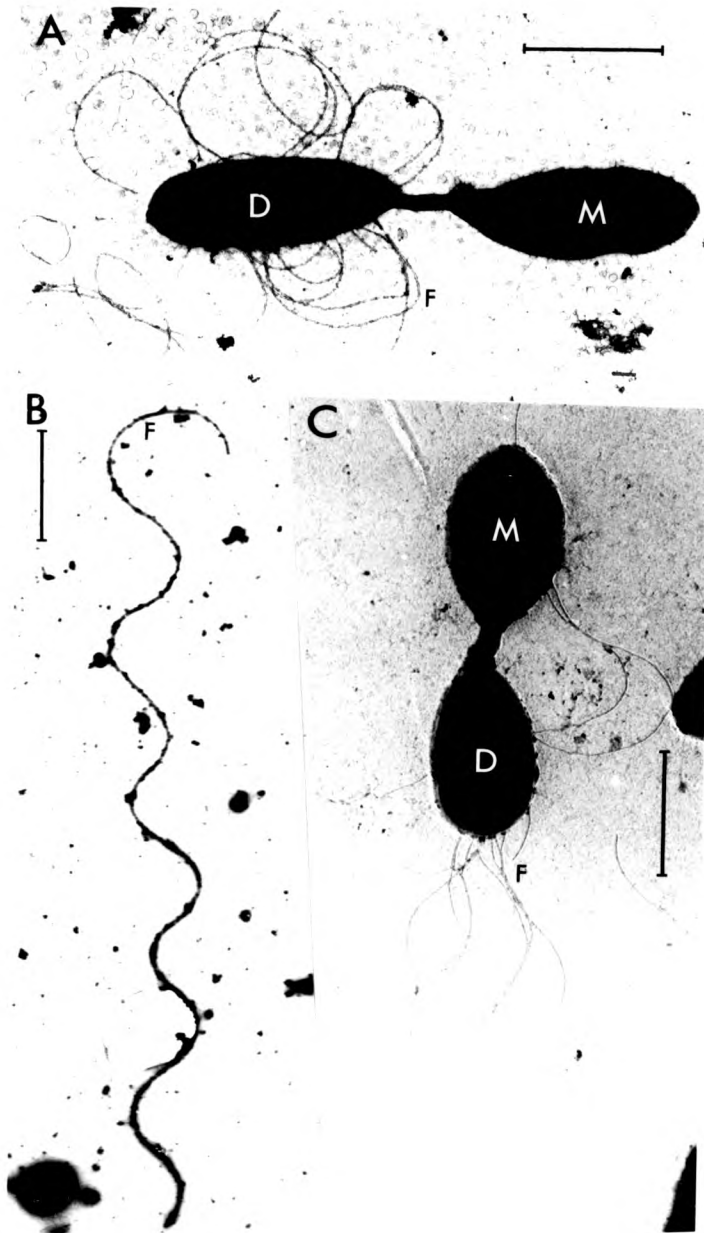
Plate 3.5   (Bars = 1 μm)

Electron micrographs

(A)   Negatively stained (uranyl acetate) mother cell (M)/daughter cell (D) duplex or 'pair', illustrating the presence of peritrichous flagella (F).

(B)   Single flagellum stained with uranyl acetate.

(C)   Gold palladium shadowed 'pair'.
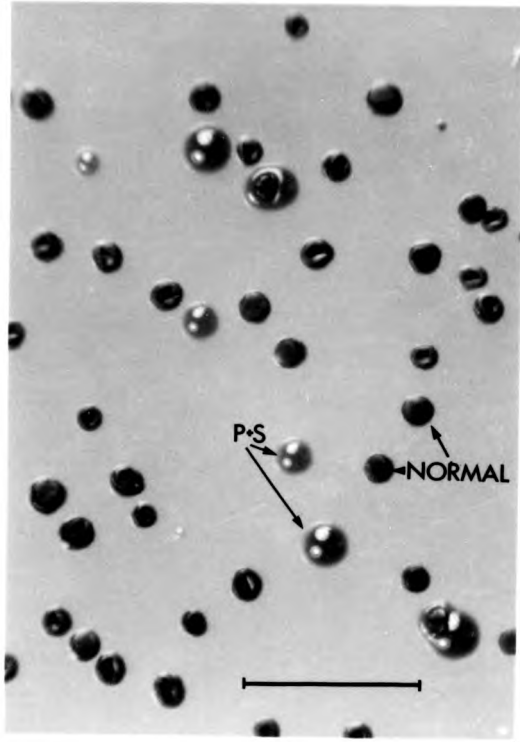
Plate 3.6

(Bar = 1 cm)

Colony morphology of P + S and 'normal' cells of Rm 5. Cells
incubated anaerobically (anaerobic bag) for 14 days at 30° C
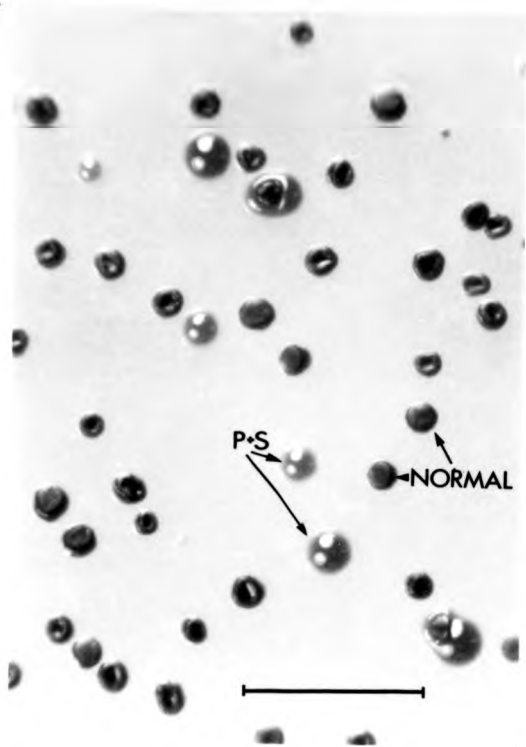with incident illumination of approximately 1000 lux.

Plate 3.6

(Bar = 1 cm)

Colony morphology of P + S and 'normal' cells of Rm 5. Cells
incubated anaerobically (anaerobic bag) for 14 days at 30° C
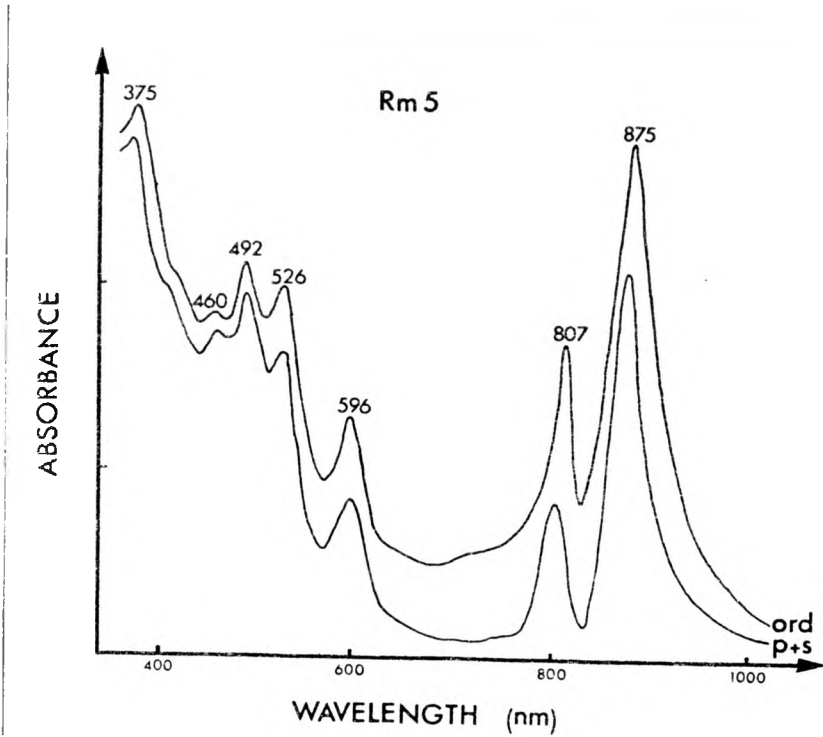with incident illumination of approximately 1000 lux.

Fig. 3.2

In vivo absorption spectra of Rhodomicrobium Rm 5 P + S cells, and
'normal' cells, grown under photosynthetic conditions. Cells were
resuspended in saturated sucrose solution.

Possible reasons why P + S colonies appear lighter colour are, firstly, the cells are less tightly packed as suggested by the mucoid nature of the colonies and secondly, they contain a higher number of swarm cells which are known to be immature and lacking in photosynthetic membranes.

P + S growth and development

As in 'normal' vegetative cell development, reproduction of P + S cells is by bud formation (i.e. terminal swelling of the filament). The daughter cell upon maturity, however, does not remain attached to the filament but is released. The mechanism is identical to swarm cell release from micro-colonies and is synonymous with binary fission. This corresponds to daughter cell release in Hyphomicrobium (Mevius, 1953) and Caulobacter (Poindexter, 1964; Terrana and Newton, 1975).

The growth and development of P + S cells has been followed by slide cultures. Slide cultures on PYR/MAL medium were prepared and inoculated with an exponentially growing P + S culture. Light (1000 lux) was provided by a 60 watt tungsten lamp and growth of the culture was followed through several generations. The generation time, from initiation of filament syntheses, through bud formation to maturity, was in the region of 6 to 8 h. Variations in the generation times were apparent and considered to be a consequence of growth, under non-ideal conditions. The oxygen tension was inconsistent, as was the light intensity which was increased when photographs were taken. (It was essential to use a green filter on the microscope, other-wise inhibition of cell growth occurred after the first generation). The behaviour of P + S cells in slide culture is shown in Plate 3.7 and Fig. 3.3. The growth sequence is also illustrated by electron micrographs (Plate 3.8). Unlike swarm cells isolated from a 'normal' vegetative culture, swarm cells from a P + S culture can only form reproductive filaments from one pole. After maturation of the swarm cell (i.e. flagella shedding, synthesis of membrane lamellae and growth), the new filament is always synthesised at the point of detachment of the swarm cell from the mother cell filament. As mentioned earlier, the mother cell and daughter cell, comprising a 'pair', have never been observed to be separated by a cross wall 'plug'.
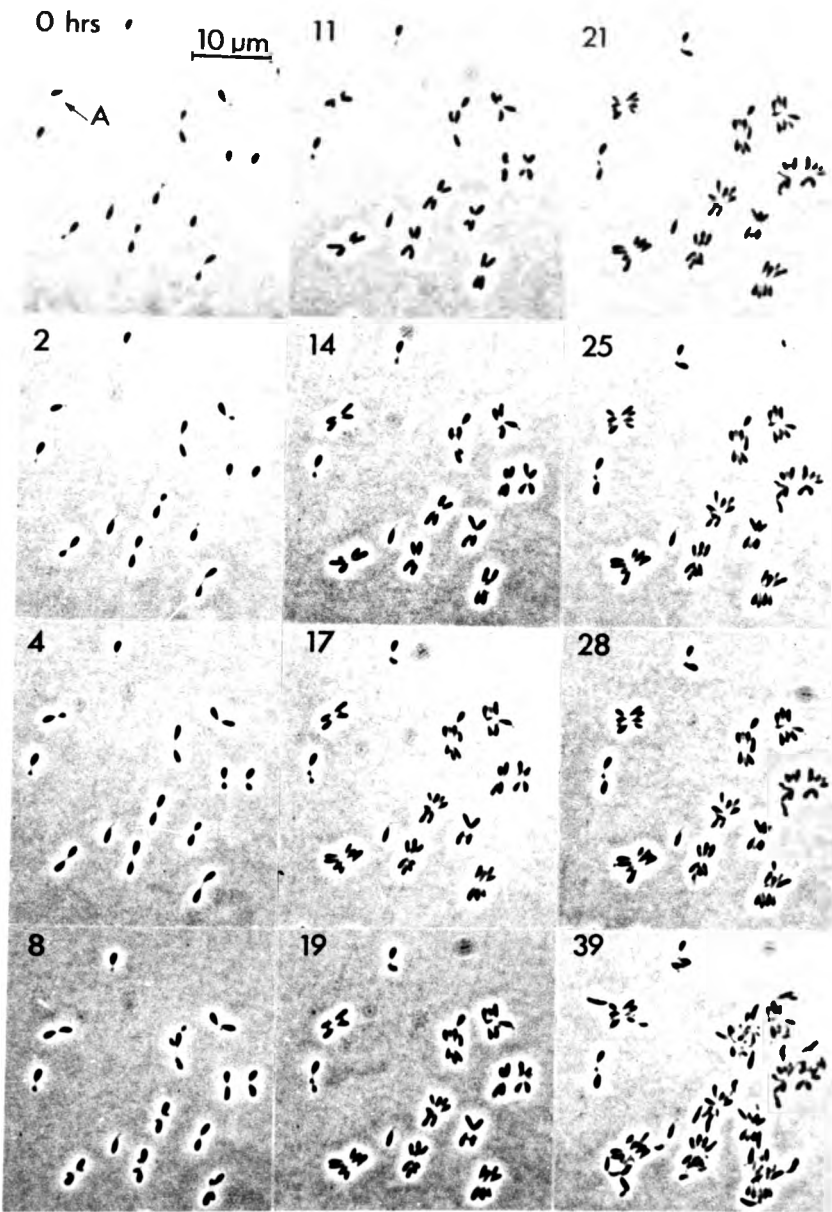
Plate 3.7

Phase contrast photomicrographs of the growth and development of
P + S cells in slide culture.
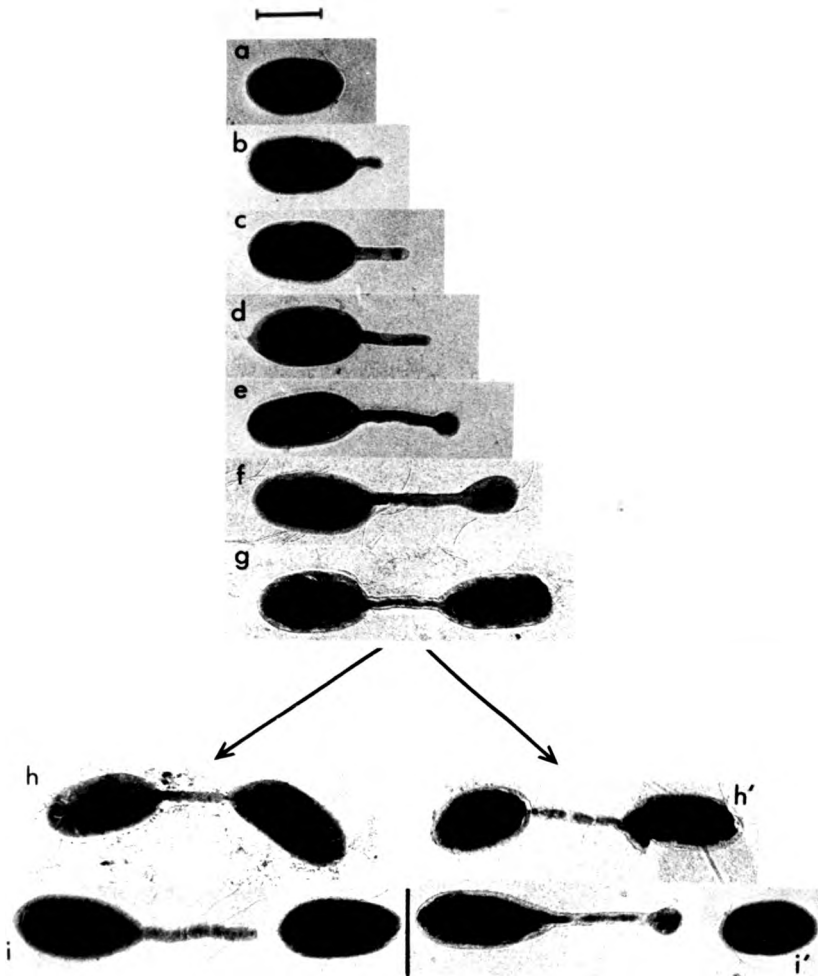
Plate 3.8 (Bar = 1 μm)

Growth sequence of simplified Rm 5 cells illustrated by electron micro-
graphs (gold-palladium shadows).

(a)   Swarm cell maturation

(b)(c)(d) Filament synthesis

(e)(f)   Bud formation and development

(g)   Daughter cell maturation. 'Pair' formation.

(h)(i)   Daughter cell separation.

(h')(i')   Initiation of new bud formation under fast growth conditions before
           release of the first swarm cell has been effected. 'Triplet formation'.

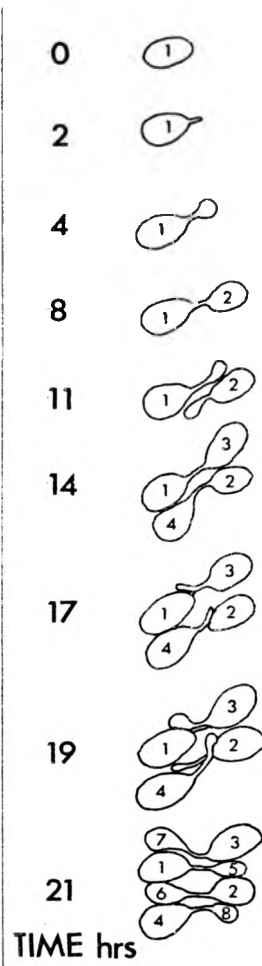Fig. 3.3

Diagrammatic representation of the growth pattern of the
Individual cell (A) illustrated in Plate 3.7, showing the
derivative cells.

Under fast growth conditions (i.e. generation times of approximately 4 h) in liquid medium, motile 'triplets' are often observed. This occurs when new bud formation is initiated by the appendaged mother cell before release of the motile swarm cell has been effected. Cultures of P + S cells have never been observed to sporulate. The induction of sporulation in these cells was attempted, by resuspending log phase P + S cells in centrifuged, cell free stale medium from a 'normal' heterogeneous culture which had sporulated. Severe disruption of the cellular division processes occurred, resulting in the formation of small and malformed cells (Plate 3.9). Exospores were not evident.

It can therefore be seen that by the use of a chemostat it is possible to obtain cells of Rm5 with unusual properties and a simplified life cycle (Fig. 3.4), i.e. under the described conditions, microcolony formation is not a prerequisite for swarm cell synthesis. This developmental cycle bears a very close resemblance to that observed in Hyphomicrobium (Leifson, 1964). It is possible to derive the simplified Rm 5 cells from a microcolony situation, since mother cells which produce swarm cells are present in all unbranched chains of cells and microcolonies, during the exponential phase of growth. Should one of these cells be excised from this position, it would establish a P+ S type situation (Fig. 3.5).

Why does the P + S situation arise, and what causes it, are two questions that have to be answered.

It has been shown (Dow, personal communication) that, during the normal vegetative growth of Rm 5 in batch culture, a mother cell is not preconditioned in its behaviour, and can give rise to either a normal daughter cell (division by 'plug' formation in a filament) or a motile swarm cell (constrictive fission with absence of 'plug' formation) or both types of cell. A mother cell, therefore, has the capacity to change its mode of division. This change of expression may be induced, as later work indicates, by environmental factors (light, pH, but more probably carbon dioxide) (Fig. 3.6). If the stimulus for swarm cell production is maintained (i.e. by growth in a chemostat) it is possible to select out cells with the simplified cycle, i.e. P + S cells.
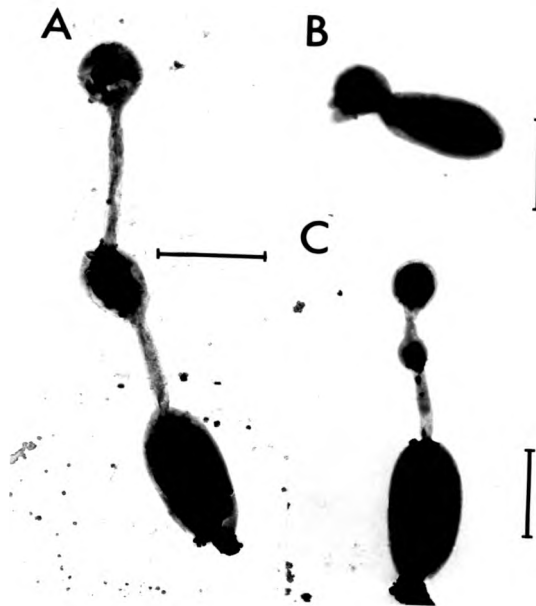
Plate 3.9

(Bar = 1 μm)

Electronmicrographs (gold palladium shadows) of small and
malformed cells induced by resuspending exponentially growing
P+ S cells in the stale growth medium which had allowed
sporulation of a 'normal' culture.
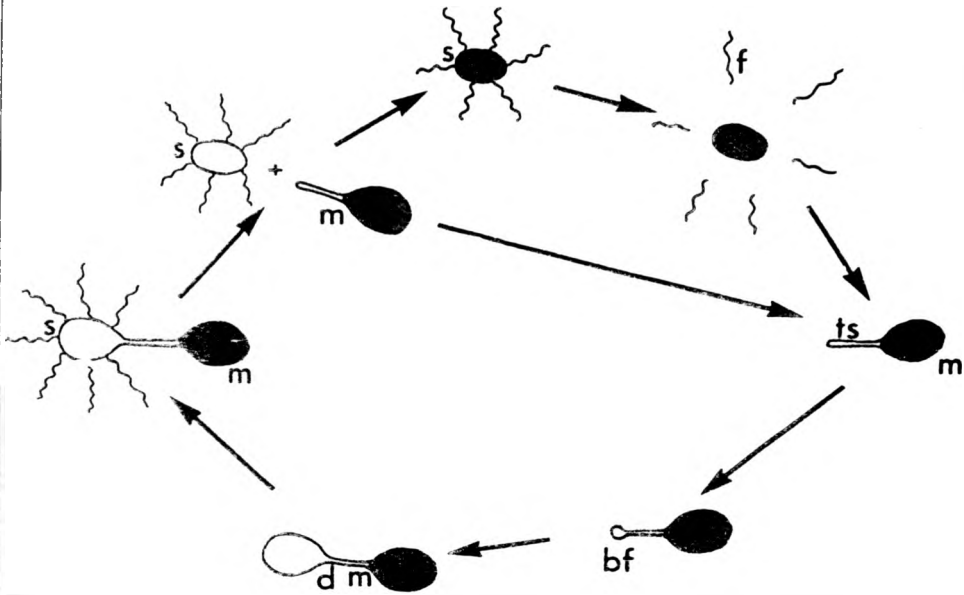
Fig.3.4

Diagrammatic representation of the Rm 5 simplified cell cycle. Swarm
cell formation in the absence of multicellular arrays.

S	swarm cell	m	mother cell
f	flagella	ts	tube (filament) synthesis
bf	bud formation	d	daughter cell
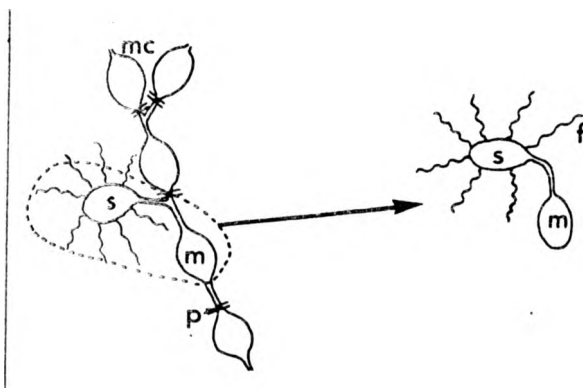
Fig. 3.5

Diagrammatic representation of the excision of a swarm cell from a
multicellular array (mc). Possible derivation of simplified cells.
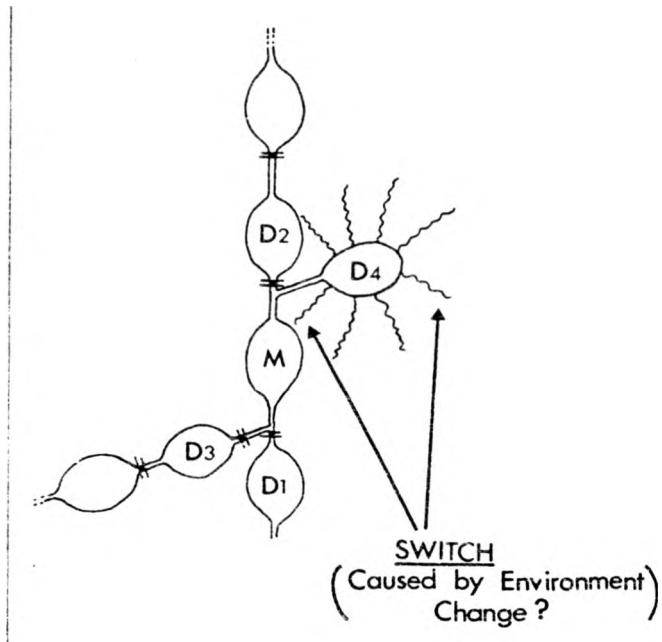
Fig. 3.6

Daughter cell (D) formation by a mother cell (M). Division processes
available.

(i) Division by 'plug' formation to give rise to normal daughter cells
   D_1, D_2, D_3.

(ii) Division by constrictive fission with the absence of 'plug' formation, D_4.

Is the mode of division influenced or regulated by environmental factors ?
(Light, pH, temperature, CO_2).

Alternatively, cells with the simplified cycle may be mutants. A mother cell which is unable to divide by plug formation because of a mutation, would be expected to give rise to P + S cells. It is also possible that the chemostat has selected out cells with a slightly faster growth rate than 'normal'.

One might expect Rm5 to behave 'normally' in a chemostat (steady state composed of microcolonies, chains and swarm cells) but there are several reasons why 'normal' vegetative cells are not ideally suited to growth under these conditions. Firstly, normal cell growth is always accompanied by considerable wall growth on the side of the culture vessel, and this has the effect of reducing the amount of light energy available to the cells in suspension. Secondly, microcolonies and chains are composed of large numbers of cells, and so the wash-out of just one microcolony in continuous culture could result in the loss of many viable units.

(b) Some characteristics of pairs and swarm (P + S) cells

Anaerobic growth in liquid medium

Rhodomicrobium has been grown on a variety of carbon sources including bicarbonate, pyruvate, malate, lactate, acetate and ethanol (Murray and Douglas, 1950; Morita and Conti, 1963; Trentini and Starr, 1967; Whittenbury and Dow, 1977).

Fig. 3.7 illustrates the growth of Rm 5 P+ S cells on the two best carbon sources, sodium pyruvate and sodium hydrogen malate. Log phase cells from a steady state chemostat were washed twice (0.1 M phosphate buffer, pH 6.8) and resuspended in fresh medium   Growth of the cells, in tightly stoppered glass cuvettes, was followed by measuring the absorbance at 540 nm.   An incubation temperature of 30° C, and an incident light intensity of 2000 lux was used. A lag phase of approximately 6 h   was observed which may be attributed to the physiological shock imposed by centrifugation and washing. As has been found for cells growing in the 'normal' cycle, malate and pyruvate were the best individual carbon sources, whilst a mixture of the two surpassed either (Table 3.1).
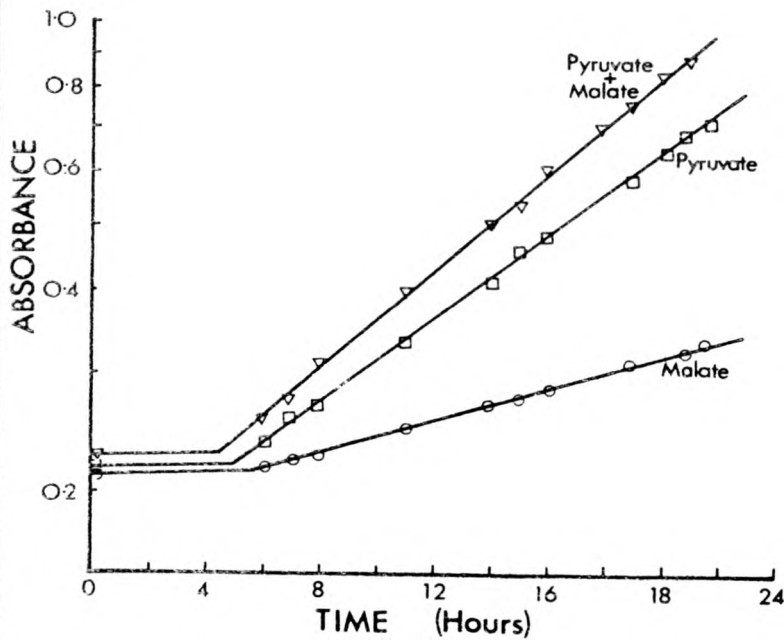
Fig. 3.7

Growth curves of Rm 5 P + S cells on sodium hydrogen malate only (O),
sodium pyruvate only (□), and a combination of sodium pyruvate and
sodium hydrogen malate. Each carbon source was at a concentration of
1.5 g/l (w/v) in mineral salts medium (30° C, 4000 lux).

Comparison of growth rates. (P + S cells versus 'normal' cells)

The growth rates of P + S cells and 'normal' vegetative cells were compared by growing the organisms under identical conditions. Two 1 litre aspirators having a common gas atmosphere (Plate 3.10, Fig. 3.8) were used for this purpose. Anaerobiosis was effected by flushing with oxygen-free nitrogen and agitation was provided by a magnetic stirrer. The temperature was maintained at 30° C and illumination was provided by a 100 watt tungsten lamp (4000 lux). To check whether the CO_2 tension had any effect on the growth rates, the experiments were repeated with 100 ml of CO_2 added to the atmospheres. Optical density (absorbance 540 nm) against time was plotted (Figs. 3.9 and 3.10), and cell doubling times estimated from the growth curves (Table 3.2). Repeat experiments gave similar results (Table 3.3).

The variation in the results is a reflection of the varying oxygen tension, a consequence of the permeability of the rubber bladder to air. Oxygen tensions of between 1% (v/v) and 4% (v/v) were apparent at the end of the experiments. However, since both cultures were subjected to the same conditions (i.e. same oxygen tensions), comparison between the two cultures in each experiment can be justified unless they differ in their oxygen sensitivity.

It can be concluded, therefore, that under these conditions P + S cells grow faster than 'normal' vegetative cells and, in addition, increasing the CO_2 tension in the culture atmosphere increases the growth rate of both cell types. The latter is further supported by the observation that, on solid media, colony growth of Rm5 is stimulated by enriching the atmosphere of the anaerobic bag with CO_2.

Effect of light intensity on growth

Growth of P + S cells at different light intensities was studied using duplicate cuvette (1 cm light path length) cultures. Log phase cells were resuspended in fresh medium, (PYR/MAL, 0.1 M phosphate buffer, pH 6.8), and their growth followed in stoppered glass cuvettes by measuring absorbance at 540 nm. A range of incident light intensities were obtained by arranging the cultures at various distances from a 100 watt tungsten lamp. Incubation was at 30° C, and excess heat from the lamp was dissipated by

TABLE 3.1

Effect of medium composition on P + S cell doubling time (30°C, 2000 lux)

Culture medium	Cell doubling time (h)
PYR/MAL	8.0
PYR	9.5
MAL	25.0

TABLE 3.2

Cell doubling times for 'normal' and P + S cells (30°C, 4000 lux)

Cells	Doubling time (h) Normal atmosphere	Doubling time (h) Enriched CO_2 atmosphere
P + S	5.0	4.7
'Normal'	5.3	5.0

TABLE 3.3

Cell doubling times for 'normal' and P + S cells. Repeat. (30°C, 4000 lux)

Cells	Doubling time (h) Normal atmosphere	Doubling time (h) Enriched CO_2 atmosphere
P + S	6.6	4.3
P + S	5.5	5.0
'Normal'	6.2	5.7

Plate 3.10

Apparatus used to compare the growth rates of Rm 5 P + S
cells and 'normal' cells. 1 litre aspirators with a common
gas atmosphere.
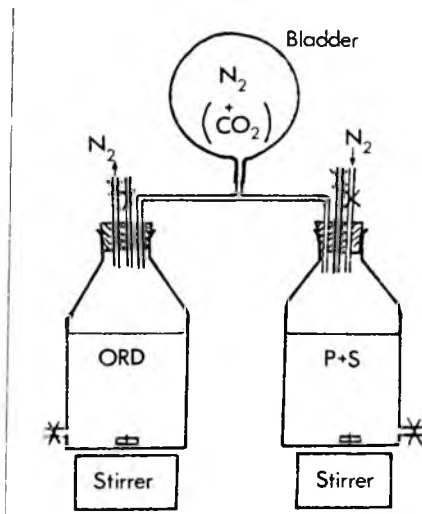
Fig. 3.8

Diagrammatic representation of Plate 3.10

Plate 3.10

Apparatus used to compare the growth rates of Rm 5 P + S
cells and 'normal' cells. 1 litre aspirators with a common
gas atmosphere.
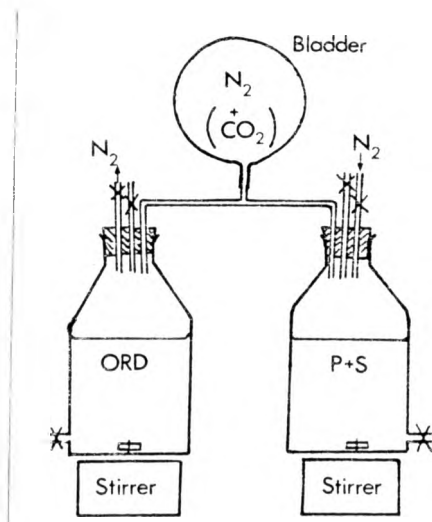
Fig. 3.8

Diagrammatic representation of Plate 3.10

Fig. 3.9

Growth curves of Rm 5 P + S cells (●) and 'normal' heterogeneous
cells (○) cultured under identical conditions (PYR/MAL medium + 0.1 M
phosphate buffer, pH 6.8, 30° C, 4000 lux).

Fig. 3.10

Growth curves of Rm 5 P + S cells (●) and 'normal' cells (○)
cultured under identical conditions (PYR/MAL medium + 0.1 M phosphate
buffer, pH 6.8, 30°C, 4000 lux).   Carbon dioxide (100 ml) was added
to the culture atmosphere.

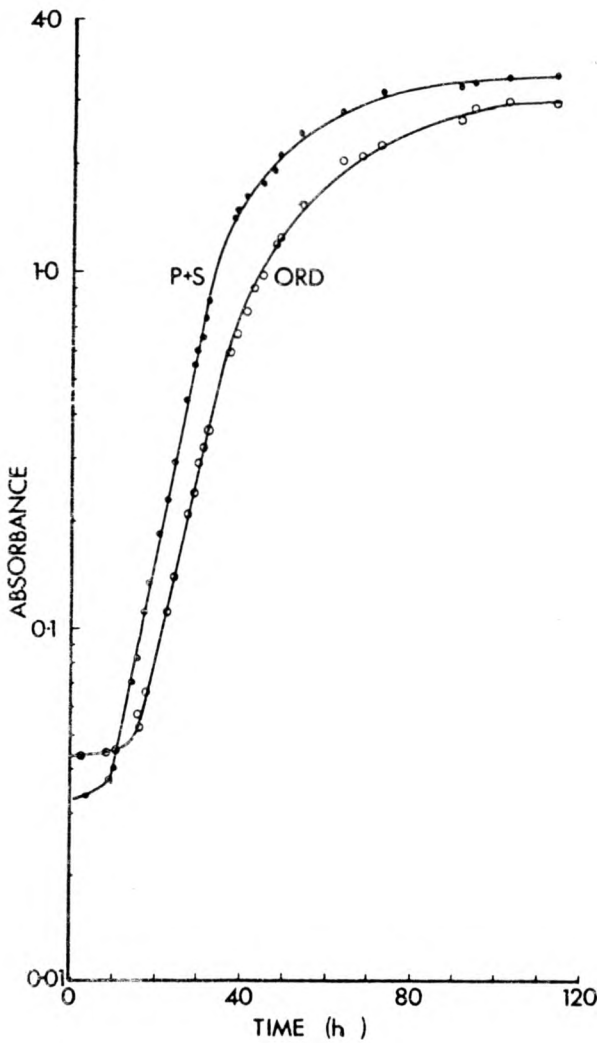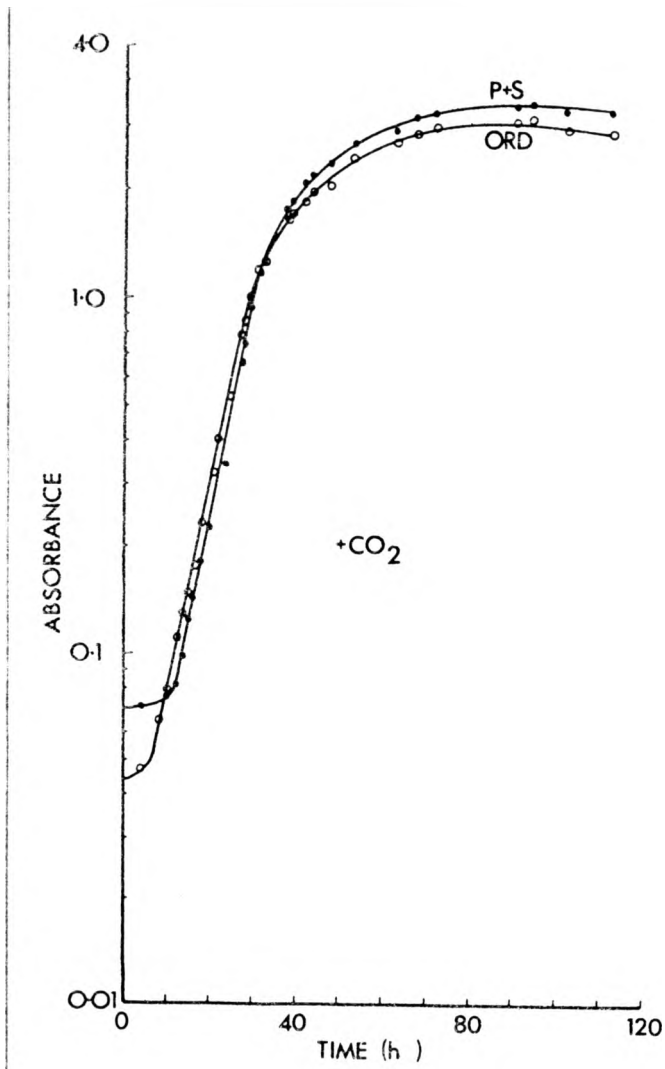an electric fan. However, due to excess heating by the illumination source, a maximum incident light intensity of 2400 lux was employed. As expected, increased illumination of the culture led to an increase in growth rate (Fig. 3.11). Fig. 3.12 shows this more clearly. Light saturation (the light intensity at which any growth rate increase becomes suppressed by an increase of illumination) has been closely approached but not reached. In Rhodopseudomonas spheroides and R. palustris this light saturation has been observed at about 10,000 lux (Sistrom, 1962a; D.Westmacott, personal communication) whilst in Rhodospirill um rubrum it is somewhat lower, at about 4000 lux (Holt and Marr, 1965).

Light limitation of P+S cell growth was therefore apparent at all the incident light intensities studied. The doubling time is approaching infinity at zero lux (i.e. the cells are unable to grow anaerobically in the dark). At 2400 lux the cells are approaching their theoretical minimum doubling time at 30° C on PYR/MAL medium. The value of this can, in theory, be extrapolated from a reciprocal plot of doubling time (DT) against light intensity (LI) (Fig. 3.13). A straight line can be drawn through the light intensity points, 1000 lux and greater (solid line). This has a linear regression coefficient of 0.947 and a slope of -0.0102, giving a theoretical minimum doubling time of $1/0.2099 = 4.9$ h . However, a straight line with a linear regression coefficient of 0.988 and a slope of -0.0480 can also be drawn through the remaining points (dotted line). Such results would indicate that below 1000 lux the doubling time is limited by a factor other than light. Later results (Section 3d) indicate that Rm 5 cells respond to a decrease in incident illumination by an increase in photosynthetic membrane content. It is possible, therefore, that in this experiment the incident light intensity of 1000 lux represents the point at which the cells have reached their maximum membrane content. Below this threshold they are unable to compensate for further light intensity reduction by additional membrane synthesis (e.g. between 2400 lux and 1000 lux the growth rate of the cells is limited by light intensity but the cells are able to compensate to a certain extent, and reduce the effect, by an increase in photosynthetic membrane content. Below 1000 lux the cells are unable to compensate for further light reduction by increasing the photopigment content).
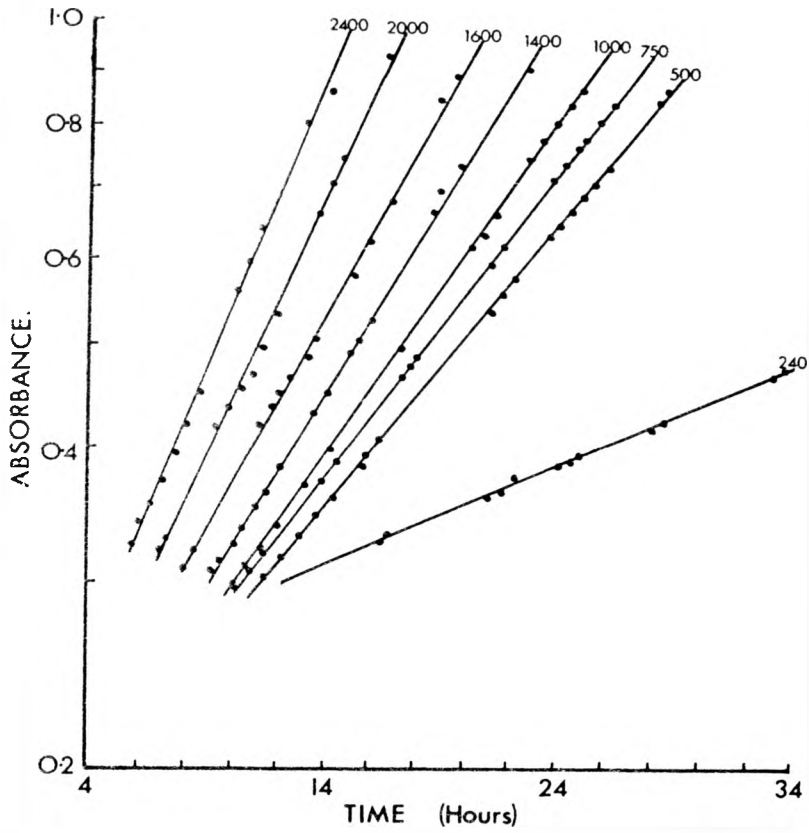
Fig. 3.11

Growth curves of Rm 5   P + S cells cultured at different incident light intensities

(PYR/MAL medium + 0.1 M phosphate buffer, pH 6.8, 30° C).
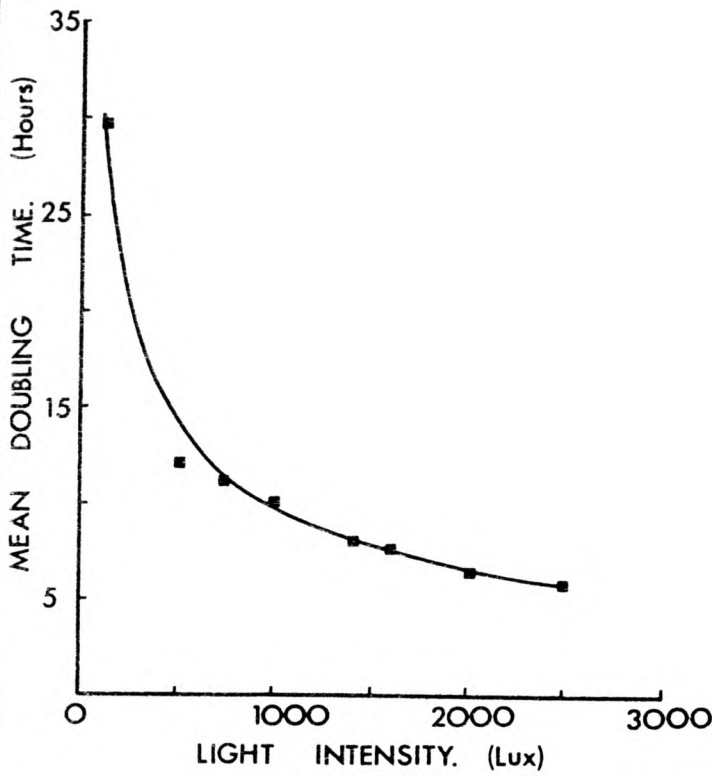
Fig. 3.12

Graph of Rm 5 P + S cell doubling time against incident light intensity.
(Doubling times were estimated from Fig. 3.11).
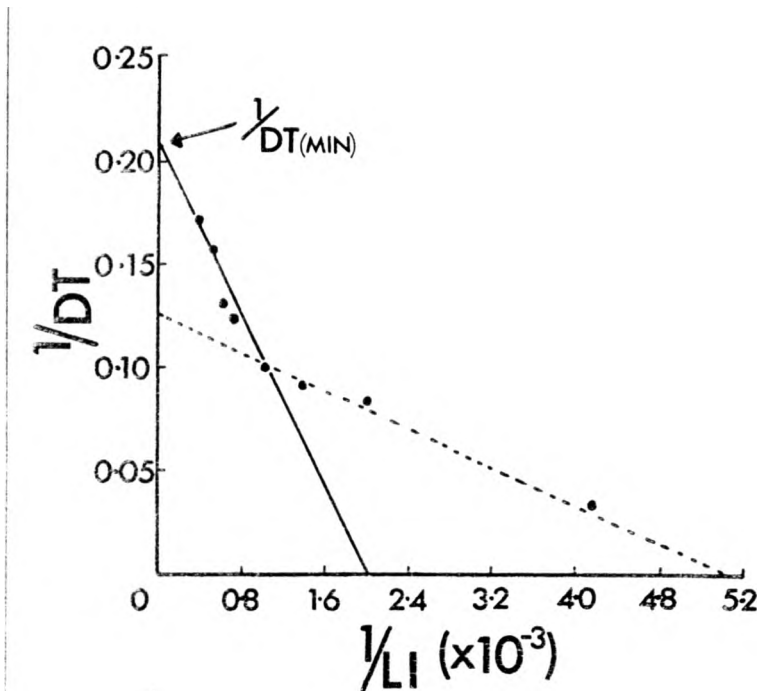
Fig. 3.13

Reciprocal plot of doubling time (DT) against incident light intensity (LI)

CO$_2$ production by P +S and 'normal' heterogeneous Rm 5 cells

The CO$_2$ production by Rm 5 growing anaerobically in the light, in 250 ml flasks, was followed.   Log phase cells (P + S and 'normal') were resuspended in 100 ml of fresh medium, (PYR/MAL + 0. 1 M phosphate buffer, pH 6. 8) the flasks being made anaerobic by flushing with oxygen free nitrogen. Incubation was at 30° C and incident illumination of 2500 lux was provided by a tungsten lamp. Samples of the gas atmospheres inside the flasks were taken at regular intervals using a 1 ml syringe, and analysed using a Pye-Unicam series 104 gas chromatograph.   Considerable differences in the CO$_2$ production curves of the two cell types is evident (Fig. 3.14).   P + S cells produced CO$_2$ throughout their batch growth,
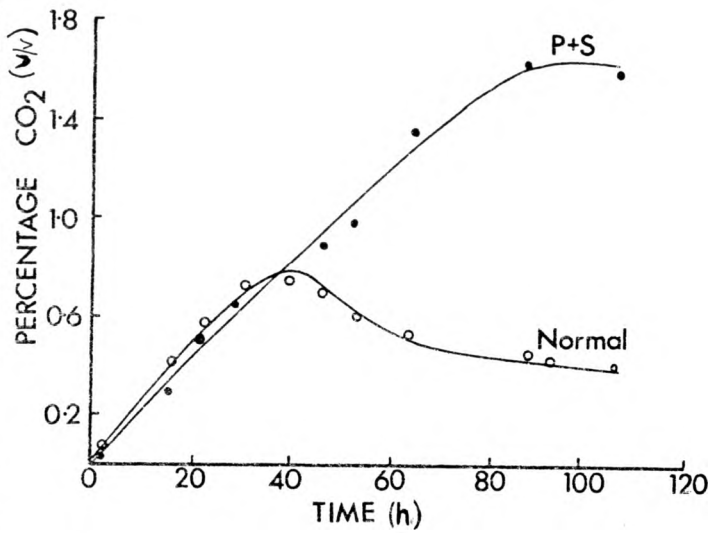
Fig. 3.14

CO$_2$ production by Rm 5 P + S cells (●) and 'normal' heterogeneous cells (○) during batch growth. (PYR/MAL medium + 0.1 M phosphate buffer, pH 6.8, 30° C, 2500 lux).

with a maximum CO_2 tension of 1.6% (v/v) being reached by stationary phase. 'Normal' cells initially produced CO_2 at a similar rate and a maximum tension of 0.8% (v/v) was reached. However, the CO_2 tension then dropped, and by stationary phase it was only 0.4% (v/v), suggesting that the cells were reutilizing some of the CO_2 produced. Similar results were obtained in the 15 litre batch culture experiments (Section 3f).

Under anaerobic conditions in the light, the reactions of cyclic photophosphorylation provide a potentially unlimited supply of ATP for the purposes of biosynthesis and thus permit an almost total assimilation of the carbon contained in the organic substrates. Little, if any, of the organic substrate is believed to be oxidised through the tricarboxylic acid (TCA) cycle which plays a minor role in the anaerobic photometabolism of most organic substrates. During the anaerobic photometabolism of organic substrates in the light, the balance between oxidation and reduction is maintained either by conversion of part of the substrate to CO_2, if it is more oxidised than the cell material, or by concomitant assimilation and reduction of CO_2, if the organic substrate is more reduced than cell material.

In the light of the results obtained, P + S cells would appear always to convert part of the substrate to CO_2, whereas 'normal' cells have to reduce and assimilate CO_2 to maintain the balance.

In point of fact, the 'difference' in the CO_2 production between the two cultures is an artifact caused by pH differences which will be fully discussed later.

Effect of carbon concentration on the growth of P + S cells
Comparison with 'normal' cells

Both P + S cells and 'normal' heterogeneous Rm 5 cells were grown in 250 ml shake flasks on medium containing different carbon concentrations (pyruvate + malate (w/w) 1:1). Log phase cells were washed twice (0.1 M phosphate buffer, pH 6.8) and resuspended in 100 ml of fresh medium containing 0.2 - 1.0 g of carbon/litre. All flasks were flushed with oxygen free nitrogen, incubated at 30° C, and illuminated by a tungsten lamp (1000 lux). At the onset of stationary phase ($\sim$ 10 days) end point

analysis of growth (dry weight determination and absorbance 540 nm), pH and CO_2 tensions (gas atmosphere) of the cultures were determined (Figs. 3.15, 3.16 and 3.17). The amount of growth of both the P + S and 'normal' cultures was directly proportional to the carbon concentration in the growth medium. Over the range of concentrations investigated, the growth of both cultures was carbon limited. However, the growth yield of P +S cells was higher than the growth yield of 'normal' cells, indicative of the former having a greater growth efficiency. It is possible that this difference could be attributed to the different stationary phase pH's of the cultures, that is, if pH limitation of the growth of normal cells was occurring. Later experiments, however, suggested that this was unlikely. Once again a difference in the CO_2 production by the two culture types was evident. This observation can be attributed to pH differences between the cultures, and will be discussed in the light of later work.

(Note. Plots of absorbance 540 nm against dry weight for both culture types are linear and identical. It can therefore be assumed that the growth of Rm 5 is truly reflected by absorbance measurements).

Effect of pH on the growth of P + S cells

Comparison with normal cells

Here the object was not to observe the growth rates of the cells at different culture pH's but to determine the pH cut off points for the growth of both cell types, i.e. the highest and lowest pH's allowing the growth of P + S and 'normal' cells. Cultures were grown anaerobically in 250 nl flasks (PYR/MA L growth medium and 0.1 M buffer) over a range of pH's. For high pH's 0.1 M Tris-HCl buffer was employed and the medium supplemented with 0.1 M sodium phosphate. All flasks were incubated at 30° C with an incident light intensity of 1000 lux. At the onset of the stationary phase (8-10 days), the optical density and pH of the culture were measured (Table 3.4). With respect to growth at different pH's, both P + S and 'normal' cells were similar. Growth was inhibited above pH 9.0 and below pH 5.5. The use of Tris-HCl buffer did not significantly impair cell growth, and as a buffer it was superior to phosphate buffer (phosphate is incorporated into the cells and the buffer breaks down). A difference between the growth efficiency and stationary phase pH of the two culture types was once again apparent.

Fig. 3.15

Growth of Rm 5 P + S cells and 'normal' heterogeneous cells on medium
containing different carbon concentrations (sodium pyruvate + sodium
hydrogen malate, 1:1(w/w). 0.1 M phosphate buffer, pH 6.8, 30° C,
1000 lux). End point analysis of biomass [(■) Dry weight P + S cells,
(□) Dry weight 'normal' cells].
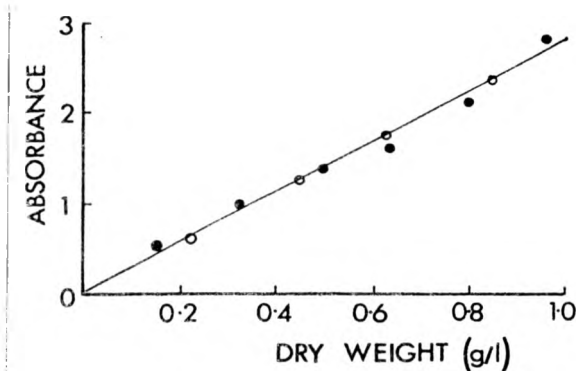
Fig. 3.16

Plots of Rm 5 absorbance 540 nm against dry weight (g/l).

(●) P + S cells

(o) 'Normal' heterogeneous cells
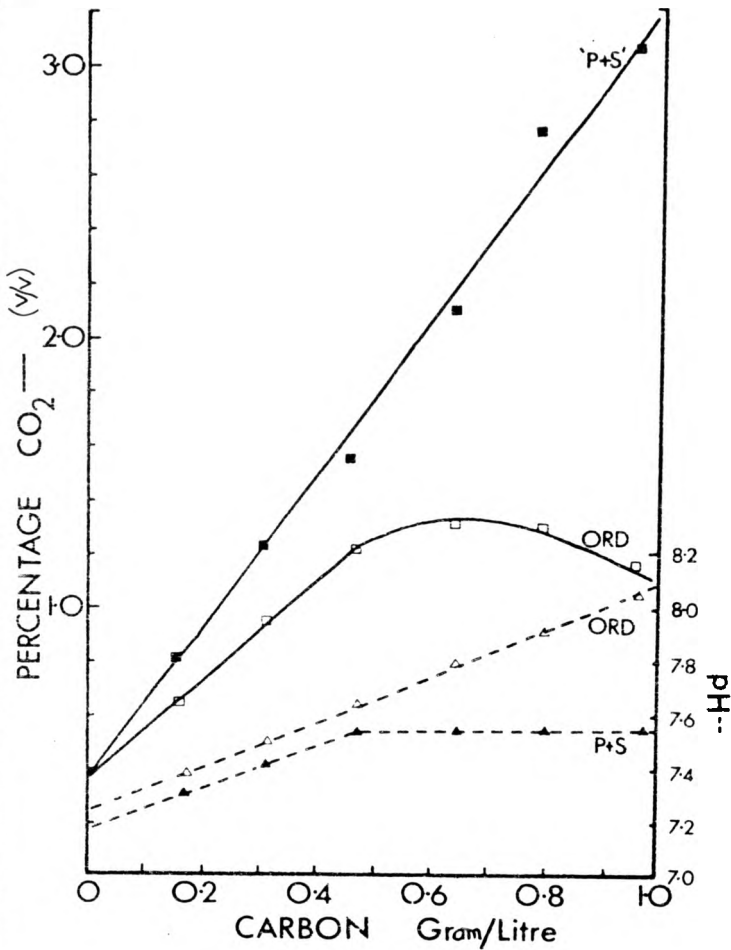
Fig. 3.17

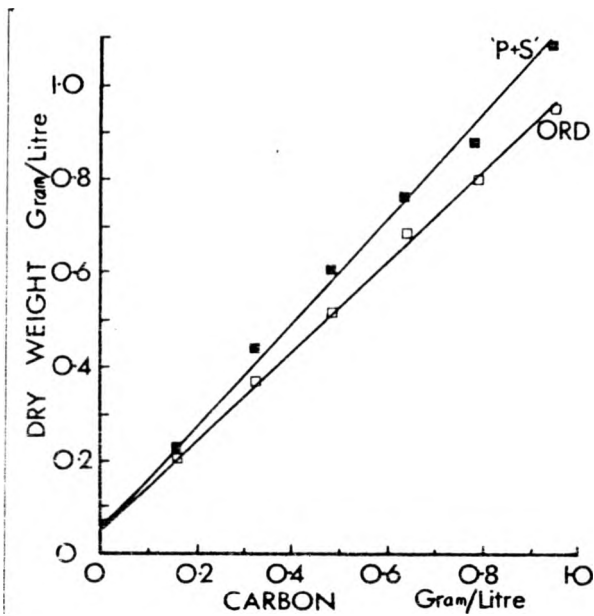Growth of <u>Rm5</u> P + S cells and 'normal' heterogeneous cells on medium containing different carbon concentrations (sodium pyruvate + sodium hydrogen malate, 1:1(w/w)). End point analysis of culture pH and gaseous CO_2 tension.

($\triangle$) pH 'normal' cells   ($\square$) CO_2 tension 'normal' cells

($\blacktriangle$) pH P + S cells   ($\blacksquare$) CO_2 tension P + S cells

TABLE 3.4

Effect of pH on the growth of P + S and 'normal cells (30° C. 1000 lux).
End point analysis.

Buffer and initial medium pH		P + S cells		'Normal' cells	
		Stationary phase absorbance 540 nm	Stationary phase pH	Stationary phase absorbance 540 nm	Stationary phase pH
Tris-HCl	9.5	0.105	8.9	0.105	9.0
Tris-HCl	9.0	1.0	8.6	1.6	8.7
Tris-HCl	8.5	2.22	8.6	1.55	8.7
PO_4	8.5	2.65	8.6	2.80	8.8
PO_4	8.0	3.0	7.8	2.85	8.7
PO_4	6.8	3.0	7.9	2.9	8.0
PO_4	6.0	3.4	7.4	2.7	7.4
PO_4	5.5	3.75	7.3	2.18	7.35
PO_4	5.0	0.075	5.25	0.045	5.25

Aerobic growth of P + S cells

Comparison with 'normal' cells

Aerobic growth in the dark by Rm 5 has been reported by Dow (1974).
Under such conditions it is likely that much of the carbon of the organic
substrate is completely oxidised to CO_2 through the reactions of the
tricarboxylic acid cycle. ATP is provided through the reactions of
oxidative phosphorylation via the cytochromes, and oxygen is the terminal
electron acceptor. This has been reported for the purple non-sulphur
bacterium R. palustris (King and Drews, 1975).

Two methods were used to examine the ability of Rm 5 P + S cells to
grow aerobically in the dark; agar plates and liquid shake cultures. Both
gave positive results. However, it required 3-4 weeks for colonies to
appear on agar plates. Such colonies were smooth and mucoid (Plate 3.11)
and considerably paler than their photosynthetic counterparts.

The subculturing of P + S cells in the dark, in aerated shake flasks
could be performed satisfactorily only twice, thereafter viability was lost.
Growth of the cells in the initial subculture was accompanied by a low
level of photopigment synthesis and resulted in a yellowy-orangey
pigmented culture. The second subculture showed little or no sign of
pigmentation and was a whitish-cream colour. The whole cell absorption
spectra of the anaerobically (light) and aerobically (dark) grown cells are
shown in Fig. 3.18. Comparison of these spectra revealed a considerable drop
in the caroteroid and bacteriochlorphyll a peaks in the aerobically grown
cultures. The spectrum of cells from the second subculture was devoid of
any significant peaks, i.e. aerobic dark growth leads to a progressive decline
in the cellular content of the bacterio-chlorophyll and carotenoids. The
inhibition of pigment synthesis in photosynthetic organisms is a consequence of the
oxygen tension and not the light. This has been demonstrated by Tauschel
and Drews (1967) working on R. palustris. A number of workers have
found that specific partial pressures of oxygen must be maintained for
optimal aerobic growth. Biedermann et al. (1967) have found that 3 to 5 mm
of mercury, partial pressure of oxygen was optimal for aerobic growth of
Rhodospirillum rubrum whilst excess aeration reduced growth. This could
account for the reduced growth and viability observed with Rm 5 P + S cells.

Plate 3.11   (Bar = 1 cm)

Colony morphology of Rm 5 P + S cells grown aerobically
at 30° C in the dark on PYR/MAL medium.   4 weeks'
incubation.
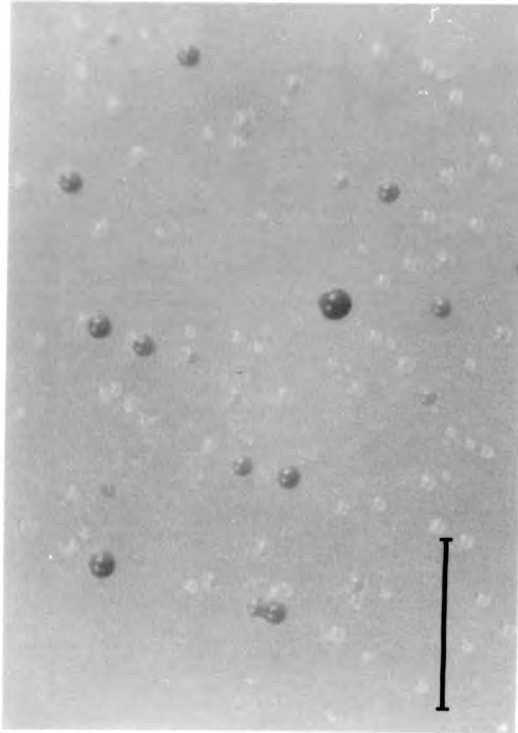
Plate 3.11   (Bar = 1 cm)

Colony morphology of Rm 5 P + S cells grown aerobically
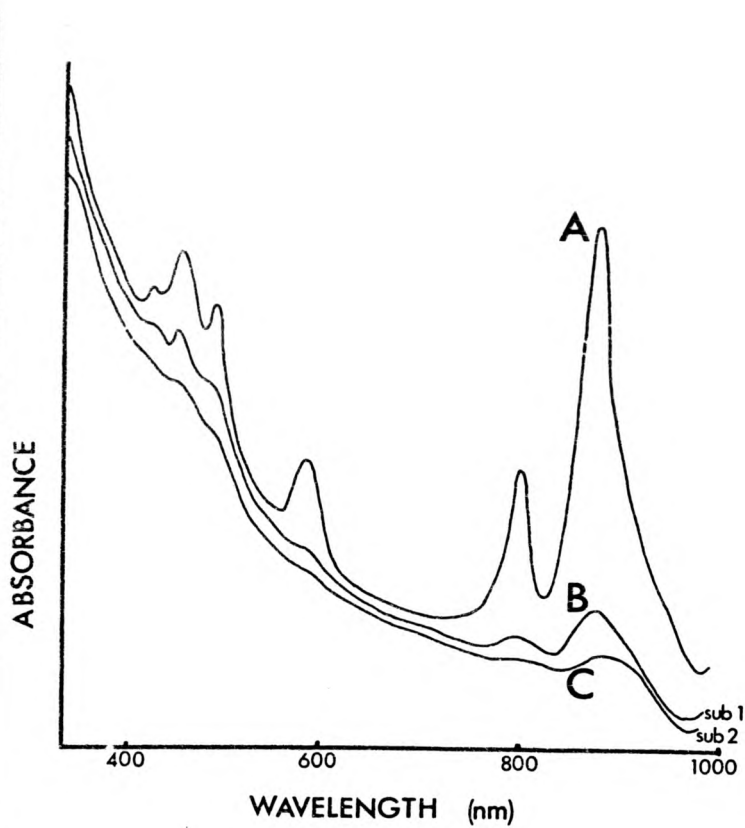at 30° C in the dark on PYR/MAL medium.   4 weeks'
incubation.

Fig. 3.18

In vivo absorption spectra of Rm 5 P + S cells resuspended in
saturated sucrose solution.

(A) Anaerobic, light grown cells.

(B) Aerobic, dark grown cells. Subculture 1.

(C) Aerobic, dark grown cells. Subculture 2.

The morphology of the cells growing under aerobic conditions was significantly affected. The initial transfer of a P + S culture growing anaerobically in the light, to aerobic dark conditions, appeared to temporarily arrest development of the swarm cells, since active motility was observed for up to 48 h after the change of conditions. A similar effect was also observed with 'normal' Rm 5 swarm cells. Subsequent development of P + S cells aerobically resulted in abnormal cell division and the appearance of cells with bizarre morphologies. -Plates 3.12, 3.13, 3.14 and 3.15 show some of the changes that occur. Interference of the division processes is apparent. The first noticeable effect would appear to be the elongation of a proportion of the cells which is closely followed by the appearance of curious lobes on the cells. "Heart' shaped cells were observed which are identical in morphology to those formed by Hyphomicrobium cells grown on methylamine (A. Lawrence, personal communication). Interference of filament syntheses was also evident, resulting in the newly formed cells being separated by little or no filament. The release of daughter cells was inhibited and clumps of cells arose. New swarm cells were not produced and all culture motility ceased within about 2 days of the initial change of conditions. Growth of the cells eventually became random, and daughter cell formation (in the loosest sense) was not confined to the poles of the cells but occurred anywhere over the cell surface. Culture viability decreased and was eventually lost.

Knobbly and unusual morphologies have also been observed in aerobically grown R. palustris (van Niel, 1944). Pfennig (1969) noted the tendency of R. acidophila to form irregular and swollen cells. In addition, Hyphomicrobium has also been observed to respond to certain environmental conditions by producing bizarre cell shapes (Tyler and Marshall, 1967; A. Lawrence, personal communication). Branch formation, and the appearance of Agrobacter cells with similar unusual morphologies was observed by Fujiwara and Fukui (1974) who correlated this change with inhibition of division and DNA synthesis.

When sections of aerobically grown P + S cells were examined, the highly organised membrane lamellae structures were considerably reduced and in many cases totally absent (Plate 3.16). In elongated cells the appearance of membrane in the cytoplasm resembling a partially formed division septum was occasionally observed.

Aerobic dark growth of 'normal' cells of Rm 5 resembled the P + S
cell growth except that interference of cell division was less pronounced.
Subculturing could be performed several times under aerobic conditions.
Cell elongation occurred, but the appearance of lobed cells was less
frequent.   Filament synthesis was affected to a lesser extent, but 'plug'
formation was randomised, i.e. some filaments had no plugs, some one,
and others two.   Plates 3.17, 3.18 and 3.19 show aerobic dark grown
normal Rm 5.
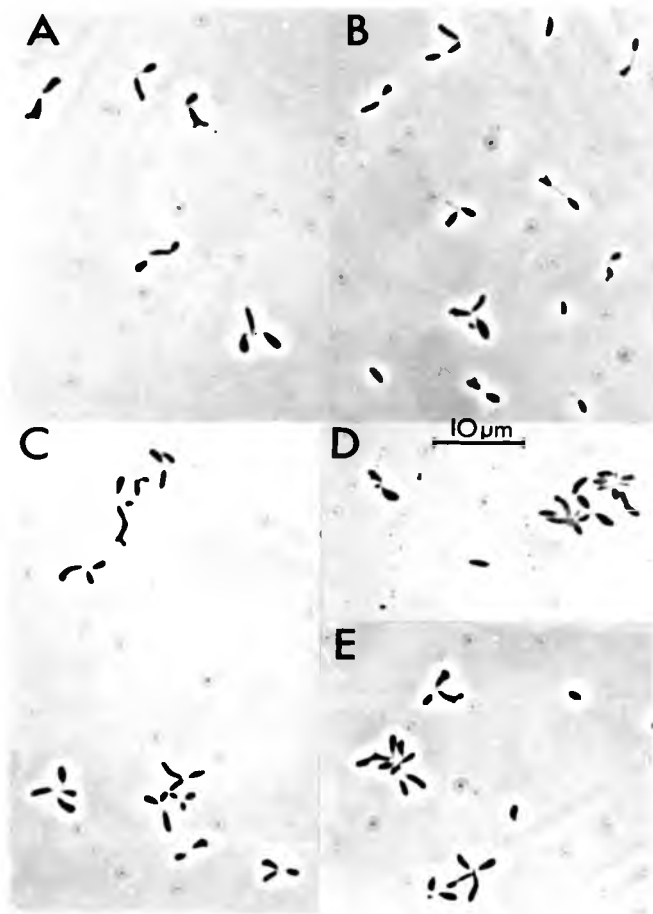
Plate 3.12

Phase contrast photomicrograph of <u>Rm 5</u> P + S cells grown   aerobically in
the dark. (PYR/MAL medium + 0.1 $\underline{M}$ phosphate buffer, pH 6.8, 30° C).

(A)(B) Early growth first subculture. Elongation of some cells and the
        appearance of lobes.

(C)   Late growth first subculture. Cell distortion and clump formation.

(D)(E) Second subculture. Random cell growth.
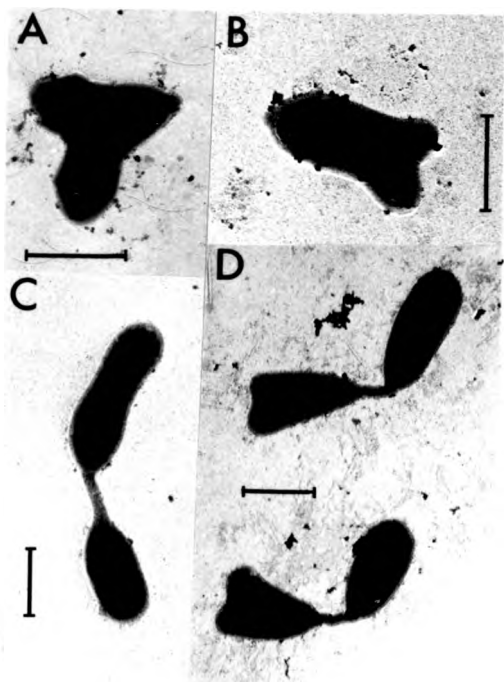
Plate 3.13   (Bars = 1 μm)

Electron micrographs (gold palladium shadows) of Rm 5 P + S cells
grown aerobically in the dark.   Interference of cell growth and division
early in the first subculture.

(A)(B)   Lobed cells with 'heart' shaped appearance.

(C)   Elongation of one of the cells in a 'pair'.

(D)   Lobe formation on one of the component cells of a 'pair'.
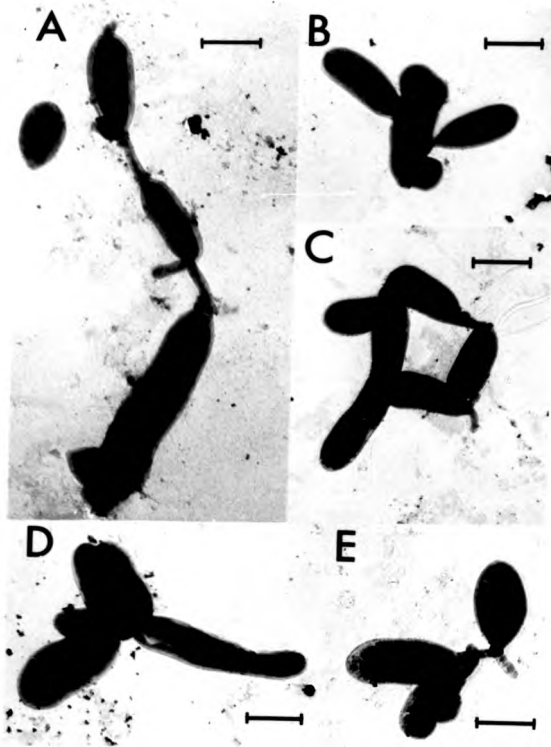
Plate 3.14   (Bars = 1 μm)

Electron micrographs (gold palladium shadows) of Rm 5 P + S
cells grown aerobically in the dark.   Cell distortion and clumping
late in the first subculture.
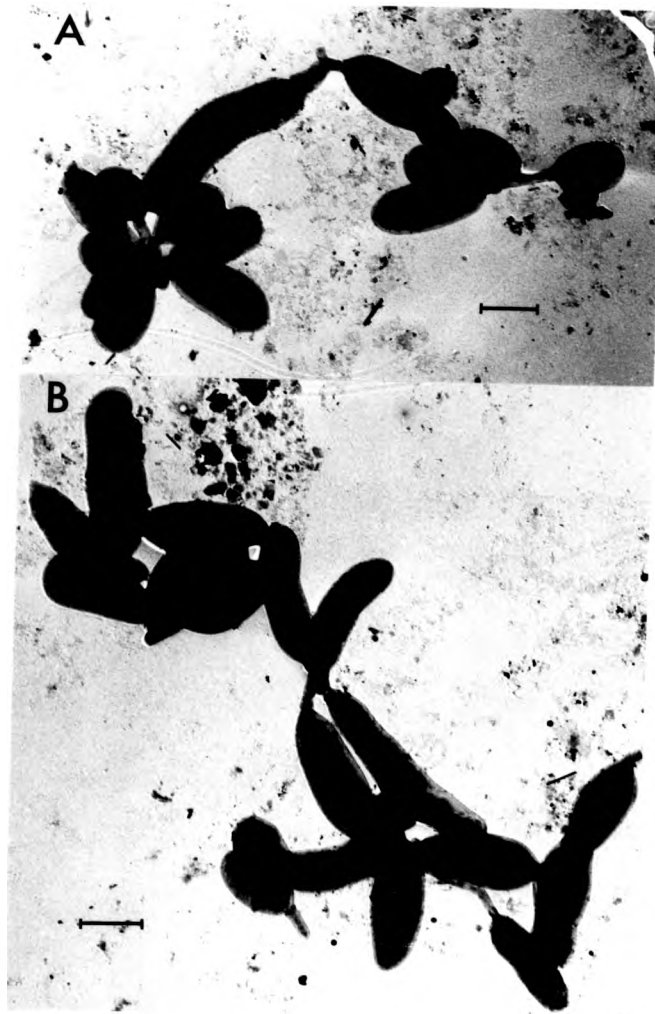
Plate 3.15   (Bars = 1 μm)

Electron micrographs (gold palladium shadows) of Rm 5   P + S cells
grown aerobically in the dark.   Cell morphology in the second
subculture.   Random cell growth and division resulting in loss of
viability.
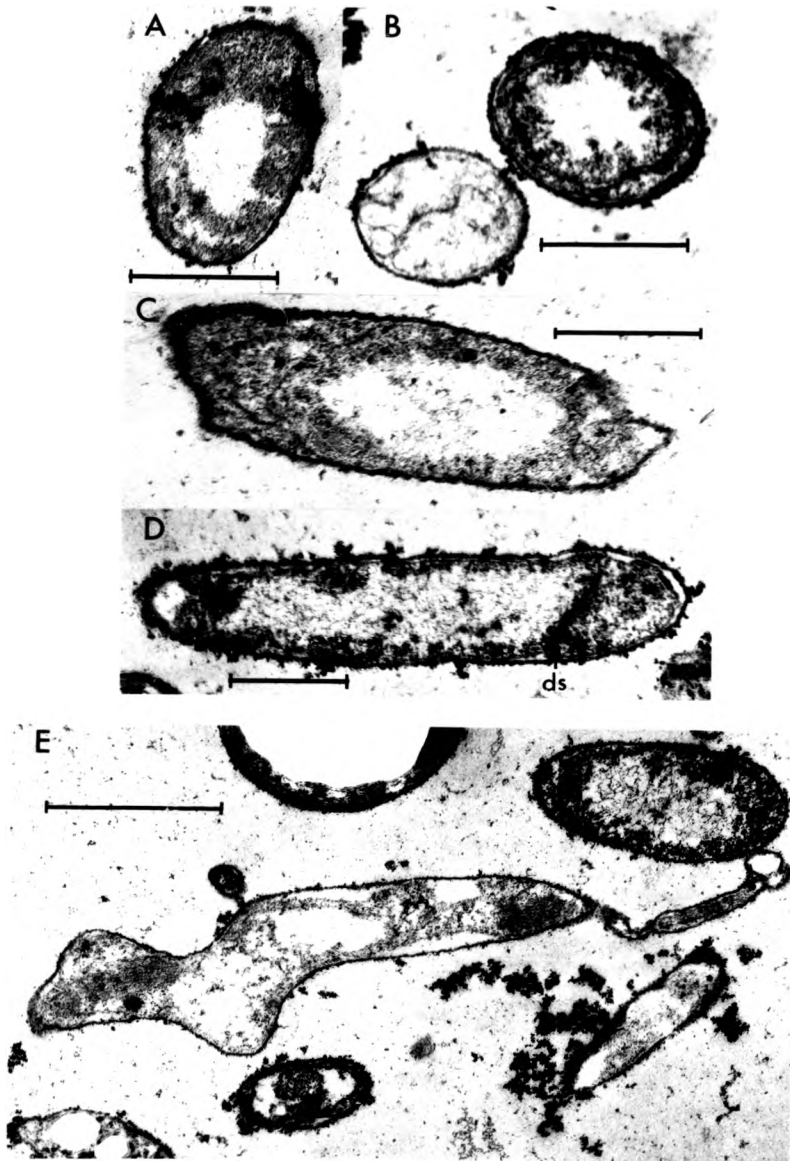
Plate 3.16   (Bars = 1 μm)

Ultra-thin sections (Kellenberger fixation) of Rm 5 P + S cells grown
aerobically in the dark.

(A)(B) Transverse sections. Membrane lamellae systems considerably
        reduced.

(C)(D) Longitudinal sections illustrating the (virtual) absence of photo-
        synthetic membranes and the appearance of a structure (ds)
        resembling a partially formed division septum.

(E)   Distorted and lobed cell. Longitudinal cell.
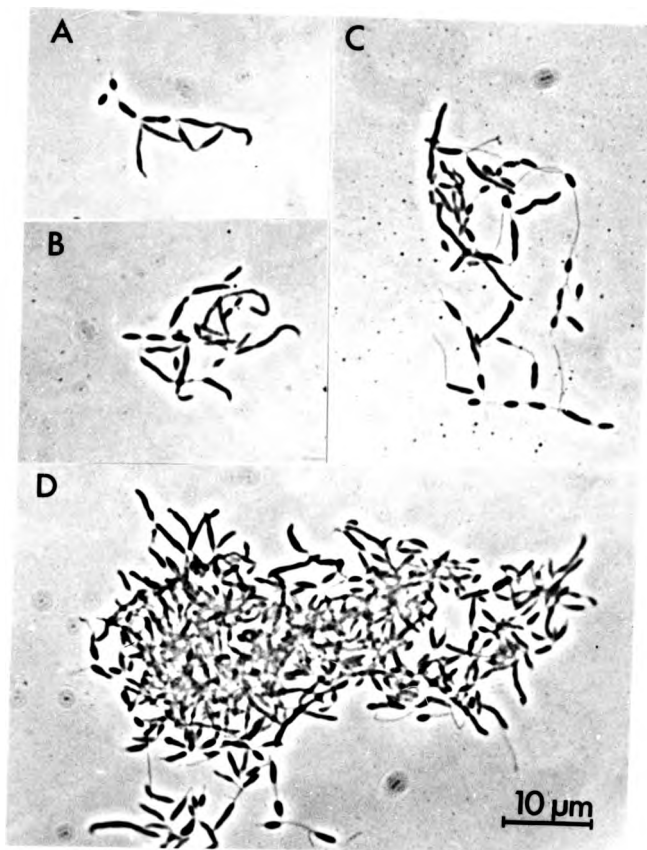
Plate 3.17

Phase contrast photomicrographs of Rm 5 'normal' heterogeneous cells growing aerobically in the dark. (PYR/MAL medium + 0.1 M phosphate buffer, pH 6.8, 30° C).
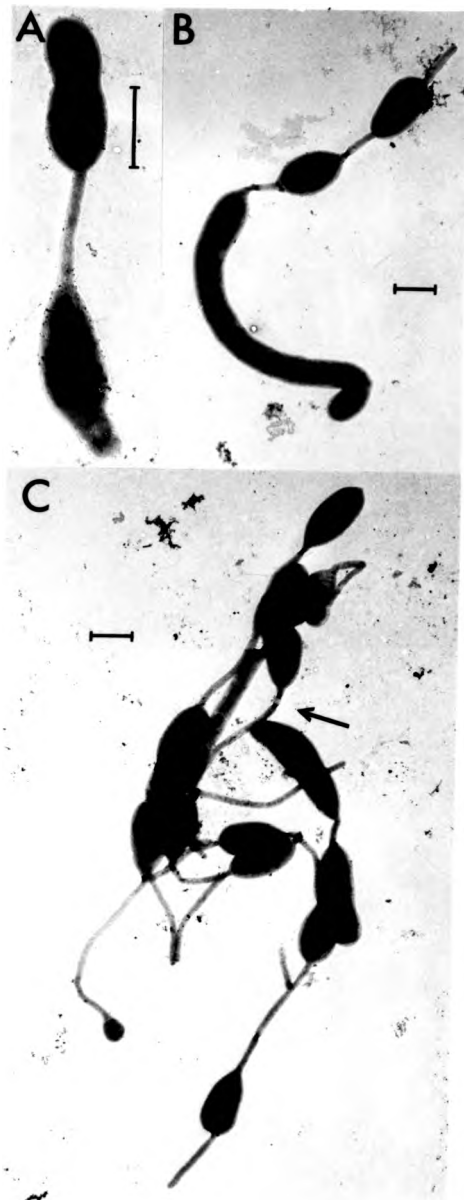
Plate 3.18 (Bars = 1 μm)

Electron micrographs (gold palladium shadows) of 'normal' heterogeneous
Rm 5 cells grown aerobically in the dark (30°C, routine medium).
Early growth.

(A)(B) Cell distortion and elongation.

(C) Multicellular array showing a filament (arrowed) with two cross
  wall 'plugs'.
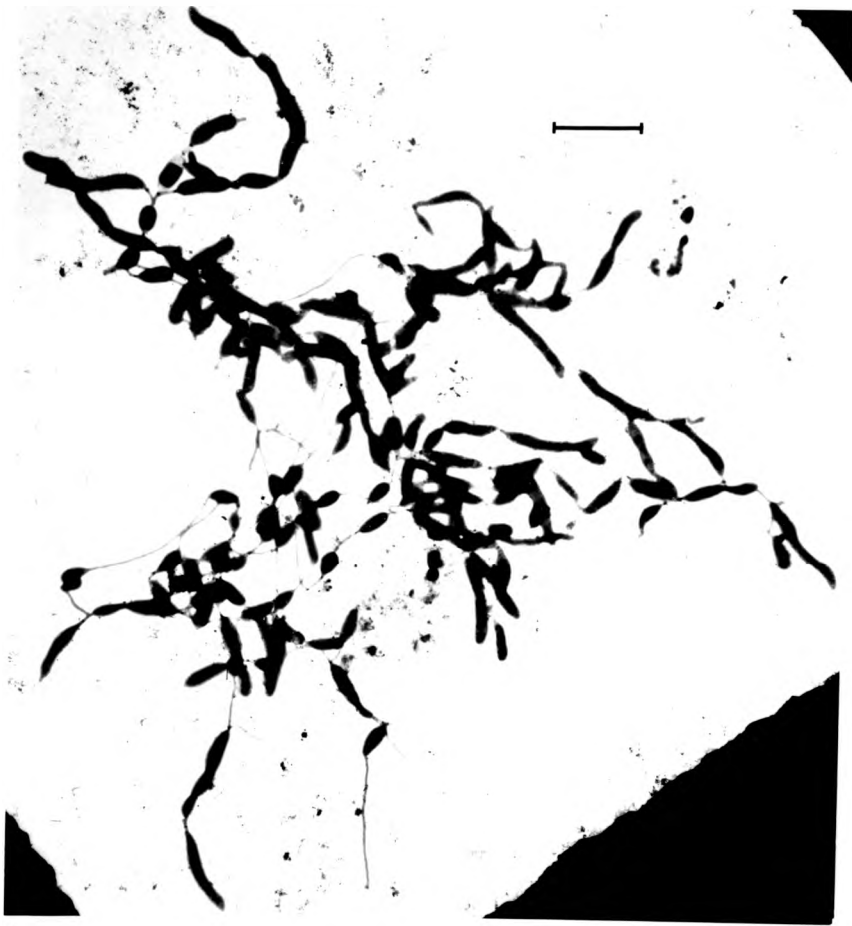
Plate 3.19 (Bar = 5 μm)

Electron micrograph (gold palladium shadow) of a microcolony composed
of 'normal' heterogeneous cells grown aerobically in the dark. Large
numbers of the cells are elongated but very few possess lobes.

(c) Polyacrylamide gel electrophoresis studies

Soluble protein fractions (S.P.F.'s) of P + S cells and 'normal cells were compared using polyacrylamide gel electrophoresis.

Cultures of P + S and 'normal' Rm 5 were grown photosynthetically (30°C, 2500 lux incident light intensity) on routine growth medium (PYR/MA L and 0.1 $\underline{M}$ phosphate buffer, pH 6.8) and harvested in the mid-exponential growth phase. Where necessary, cells were labelled with ^{35}S-methionine for 3 h prior to harvesting. Swarm cells in the 'normal' culture were separated from the multicellular arrays (microcolonies) using the glass wool column technique. The column was then washed thoroughly (0.1 $\underline{M}$ phosphate buffer, pH 6.8) to release trapped swarm cells. The glass wool was then removed from the column and the microcolonies resuspended in a small volume of buffer (0.1 $\underline{M}$ phosphate, pH 6.8). All cells were broken using a 'French Press', and the S.P.F.'s of P + S cells (P + S), 'normal' microcolonies (MC) and 'normal' swarm cells (SW) prepared.

The three soluble protein extracts were then compared using a variety of polyacrylamide gel electrophoresis techniques.

(i) Disc gels 6% (w/v) acrylamide

The results obtained using this technique were poorer than anticipated (Plate 3.20; Fig. 3.19) and significant differences between the extracts were difficult to detect due to inadequate resolution, and diffusion of the bands in the lower half of the gel.

The electrophoretic protein profiles of the swarm cell extract and the microcolony extract appear virtually identical although slight differences in the intensities of some of the bands were evident. In comparison the profile of the P + S extract appeared to differ greatly in the bands of lower molecular weight. However, since these differences occur in the lower region of the gels, where the diffusion of the protein molecules and spread of the bands is greatest, their significance is difficult to justify.

(ii) Slab gels (10% (w/v)-30% (w/v) acrylamide exponential gradient)

The results obtained with this system were a considerable improvement on those obtained with disc gels (Plates 3.21, 3.22; Fig. 3.20). Direct comparison between the extracts was easier and more precise since all the

(c) Polyacrylamide gel electrophoresis studies

Soluble protein fractions (S.P.F.'s) of P + S cells and 'normal cells were compared using polyacrylamide gel electrophoresis.

Cultures of P + S and 'normal' Rm 5 were grown photosynthetically (30° C, 250u lux incident light intensity) on routine growth medium (PYR/MAL and 0.1 M phosphate buffer, pH 6.8) and harvested in the mid-exponential growth phase. Where necessary, cells were labelled with ^{35}S-methionine for 3 h prior to harvesting. Swarm cells in the 'normal' culture were separated from the multicellular arrays (microcolonies) using the glass wool column technique. The column was then washed thoroughly (0.1 M phosphate buffer, pH 6.8) to release trapped swarm cells. The glass wool was then removed from the column and the microcolonies resuspended in a small volume of buffer (0.1 M phosphate, pH 6.8). All cells were broken using a 'French Press', and the S.P.F.'s of P + S cells (P + S), 'normal' microcolonies (MC) and 'normal' swarm cells (SW) prepared.

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Plate 3. 20

Disc gel electrophoresis (6%(w/v) acrylamide) of the soluble protein
fractions (S.P.F.'s) of photosynthetically grown Rm 5   P + S cells, 'normal'
swarm cells (Sw) and 'normal' multicellular arrays (Mc).
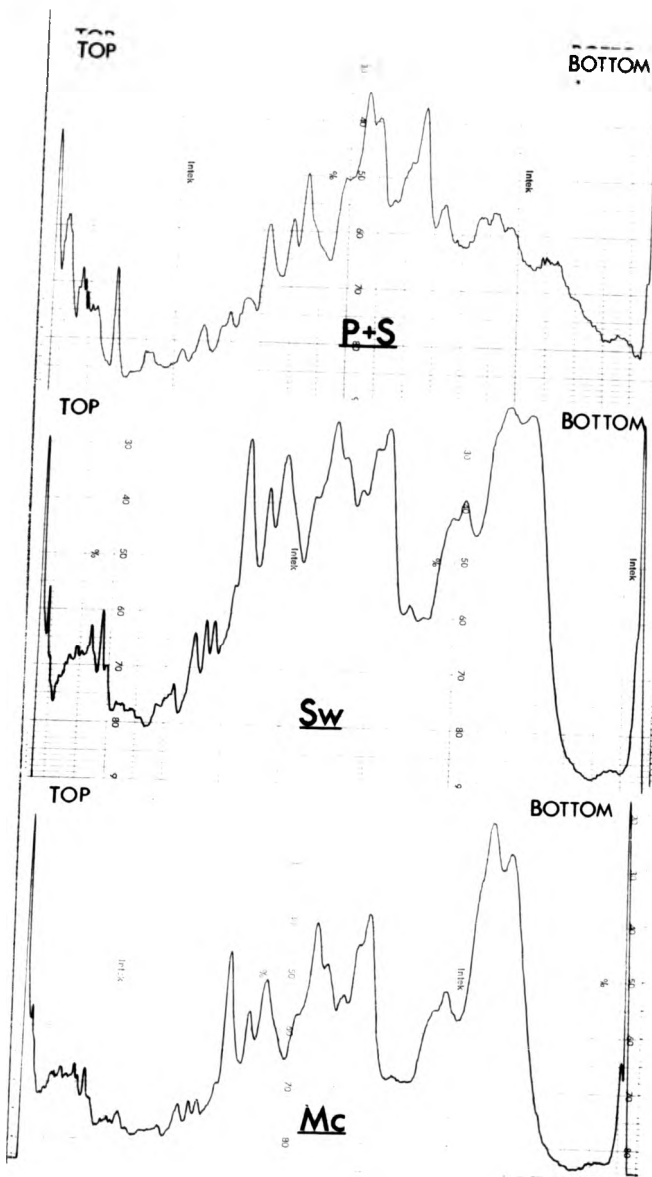
Fig. 3.19

Densitometric scans (Gilford spectrophotometer) of the SPF disc
gels (Plate 3.20).

P + S = 'P + S' cell extract

Sw   = 'Normal' swarm cell extract

Mc   = 'Normal' multicellular array extract
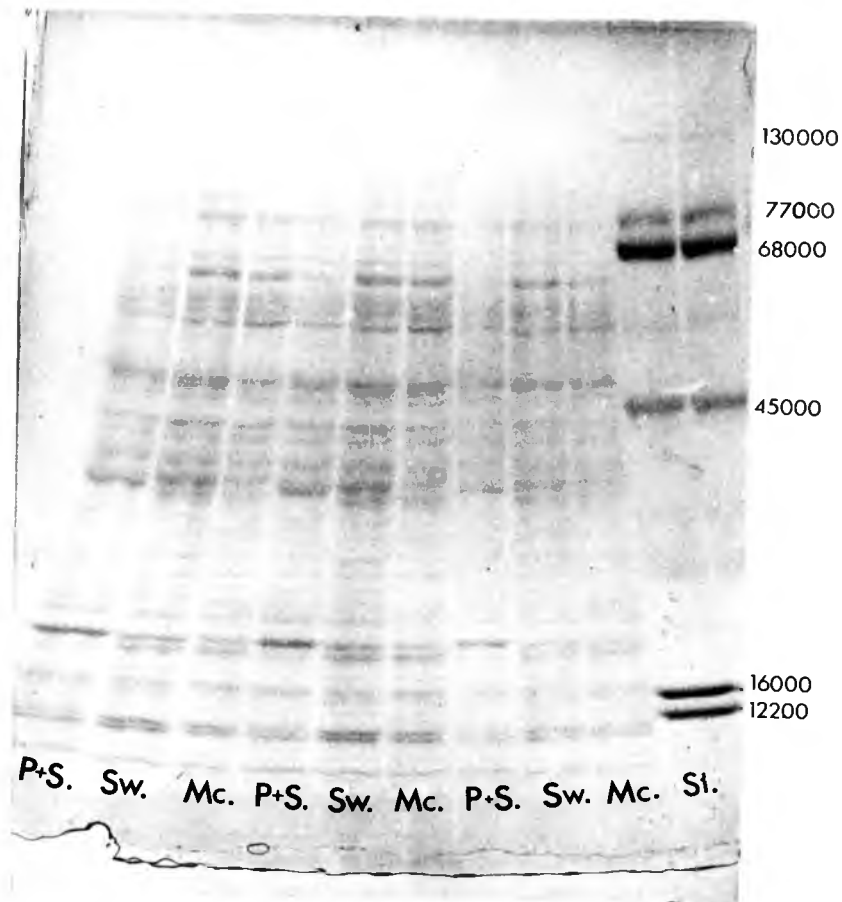
Plate 3.21

Slab gel of the SPF's of the different Rm 5 cell types grown photo-
synthetically. The gel was stained $(0.1\% \, (w/v) \, \text{Coomassie blue})$ and
destained as described in Section 2. Band intensity differences
between the cell types are apparent in the lower half of the gel.

P+S. Sw. Mc. P+S. Sw. Mc. P+S. Sw. Mc.

X→

Y→

Z→
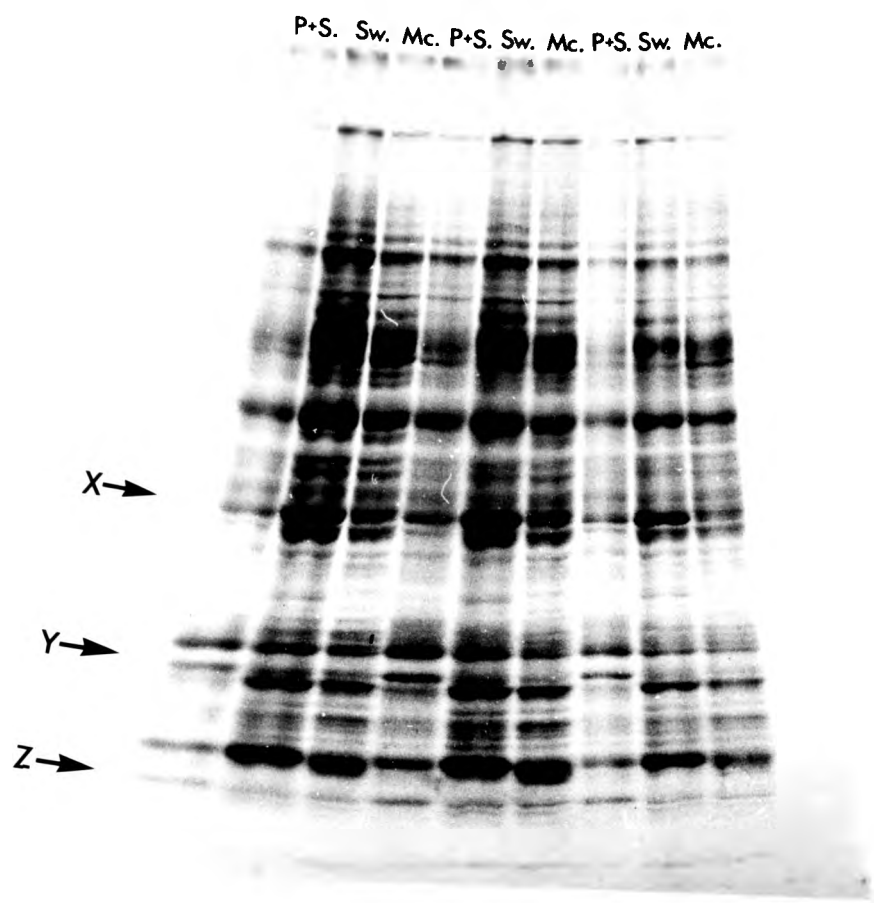
Plate 3.22

Autoradiograph of the slab gel of the SPF's of the different Rm 5 cell types (Plate 3.21).   Exposure time 14 days.

X   Band enhanced in Sw extract

Y
Z   Bands enhanced in P + S extract
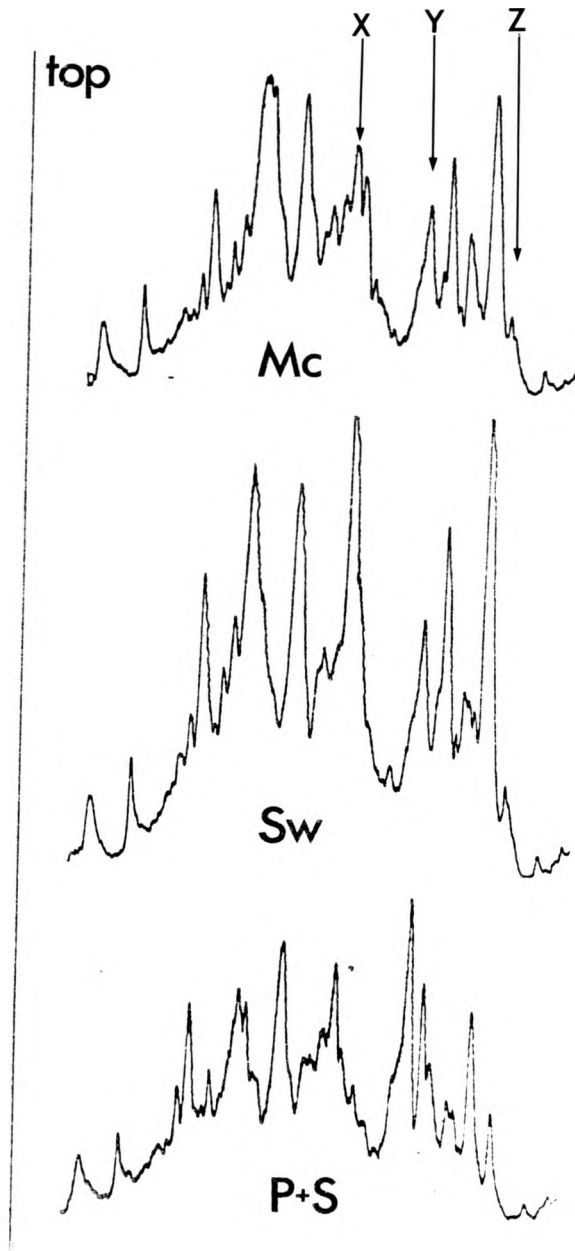
Fig. 3.20

Densitometric scans (Joyce Loebyl densitometer) of the slab
gel autoradiograph (Plate 3.22). P+S, Mc and Sw tracks.
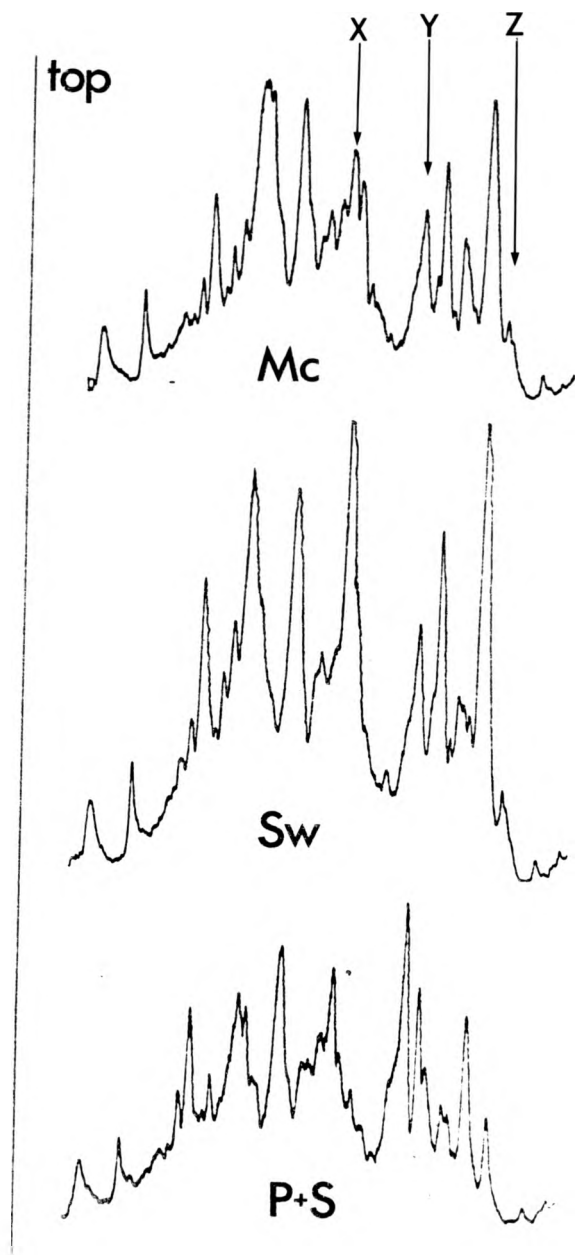
Fig. 3.20

Densitometric scans (Joyce Loebyl densitometer) of the slab
gel autoradiograph (Plate 3.22). P+S, Mc and Sw tracks.
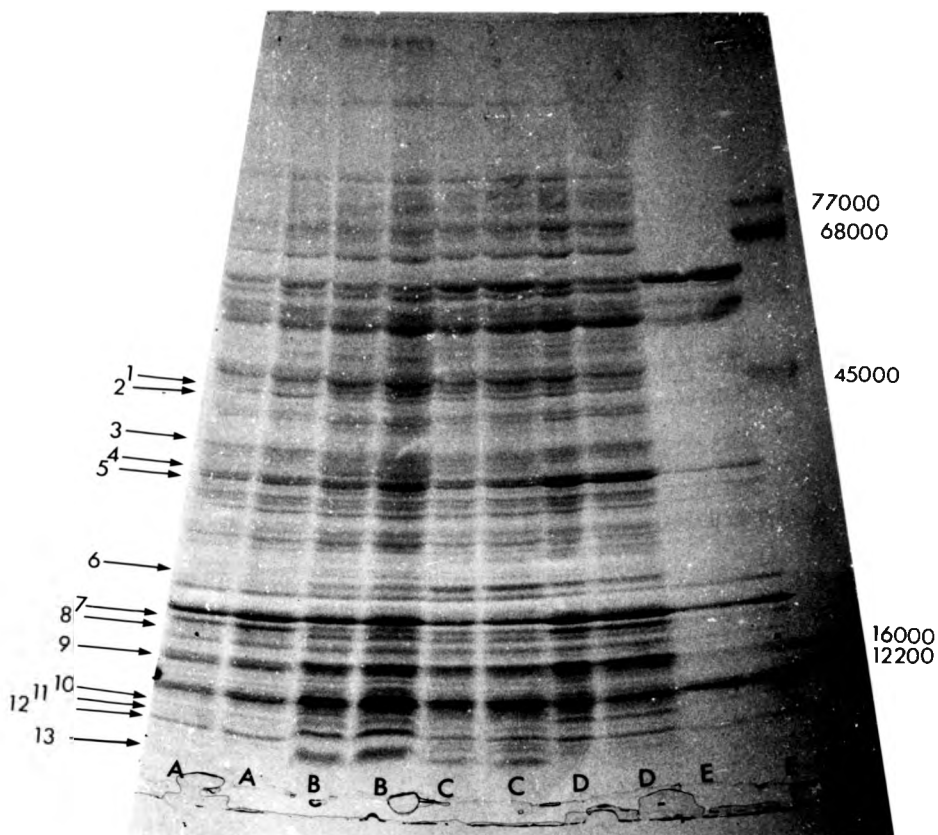
Plate 3.23

Slab gel of P + S and 'normal' heterogeneous Rm 5 SPF.'s. The cells were either grown aerobically in the dark or photosynthetically.

(A) 'Normal' heterogeneous cells (Mc + Sw). Anaerobic, light grown.

(B) P + S cells. Anaerobic, light grown.

(C) P + S cells. Aerobic, dark grown.

(D) 'Normal' heterogeneous cells (Mc + Sw). Aerobic dark grown.

(E) A 'reverting'culture of P + S cells, i.e. P + S,+Mc + Sw. The cells were grown anaerobically in the light on medium containing CO_2 and ethanol as carbon sources.

(m) Molecular weight markers.

SPF's could be run together on the same gel under identical conditions. The use of an exponential gradient greatly improved resolution, especially in the lower half of the gel, where diffusion of the low molecular weight proteins and band spread was minimised. By using ^{35}S methionine labelled extracts, and autoradiography, sensitivity was greatly increased.

All the SPF's gave similar electrophoretic protein profiles and no bands present on one profile were absent on either of the other two. However, considerable differences in the intensities of some of the bands were evident between the extracts. For example, the intensity of band (X) is greatly enhanced in the swarm cell SPF in comparison to the other two, whereas bands (Y) and (Z) are markedly enhanced in the P + S SPF. All major differences appear to be confined to the proteins of lower molecular weight (less than 45,000 daltons).

Unlabelled SPF's of P + S and 'normal' cells grown aerobically in the dark (1st subculture) were also run on slab gels and compared with those of cells grown anaerobically in the light. Growth of a 'normal' culture under aerobic conditions did not permit separation of the cells into swarmers and microcolonies. Consequently, a heterogeneous extract was prepared containing both cell types (MC + SW). A heterogeneous extract of cells growing photosynthetically was also prepared for comparison. The change of growth conditions (anaerobic light to aerobic dark) appeared to have little effect on the gel patterns (Plate 3.23) apart from slight differences in the intensities of some of the low molecular weight bands. A number of differences are evident between the SPF's of normal cells and P + S cells grown under either condition. These differences would appear to be magnified by the use of a heterogeneous extract of 'normal' cells. Under anaerobic conditions the intensities of bands 2, 5, 7 and 12 are enhanced in 'normal' cells whereas the intensities of bands 1, 4, 6, 8, 11 and 13 are enhanced in P + S cells. Under anaerobic conditions, the differences are less pronounced but the intensities of bands 5, 7, 9 and 10 appear to be enhanced in the 'normal' cells.

(iii) Two dimensional gels

1st dimension, 30% $\frac{(w/v)}{}$ acrylamide. Separation by isoelectric focussing.

2nd dimension, 10% $\frac{(w/v)}{}$/- 30% $\frac{(w/v)}{}$/acrylamide. SDS. electrophoresis.

The slab gel technique was successful in so far as it identified gross differences between the S.P.F.'s of the Rm 5 cell types. To improve the analysis with respect to resolution and identification of single polypeptides, two dimensional gels were prepared. All gels were run using ^{35}S methionine extracts and autoradiographed (Plates 3.24, 3.25, 3.26).

Although highly complex spot patterns were obtained, the high reproducibility permitted spots on one separation to be correlated with those obtained from a separate analysis. In addition to differences in spot intensities between the S.P.F.'s of Rm 5 cell types, some polypeptides were absent, and these have been indicated in plates 3.24A, 3.25A and 3.26A, i.e. there are at least 13 polypeptides present on the SW separation which are completely absent from the MC pattern (e.g. SW F19). Conversely there are at least 7 polypeptides present on the MC pattern which are completely absent from that of the SW (e.g. MC Q16).

Unfortunately the P + S extract was underlabelled and it is not possible to determine which spots present on the MC and SW separations are absent from the P + S separation. However, at least 2 polypeptides present on the P + S separation are absent from the MC and SW separations.

Of the gel techniques employed to compare the soluble proteins of the different Rm 5 cell types this was undoubtedly the best, permitting the detection of single polypeptide differences in the extracts. Further work is required to characterise the separations and to identify the significant polypeptides (i.e. flagellin, Ribulose bis phosphate carboxylase, RNA polymerase).

A mutant was isolated which expressed the simplified cell cycle in continuous culture under conditions of high CO_2 tension but which lost viability when the CO_2 tension was reduced. Under such conditions, cells with unusual morphology were apparent (Plate 3.27), and characteristic cell clumps rather than microcolonies produced. Tube synthesis was confined to one pole only, and all new filament synthesis, branching and bud formation, appeared to be confined to the prostheca separating the mother and daughter cells of the pairs (Fig. 3.21). Viability was eventually lost, due probably to filament rupture caused by physical over-crowding of the cells. Such an effect is likely to be caused by mutation(s) in

FIRST DIMENSION

SECOND DIMENSION

P+S

1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25

A B C D E F G H I J K L M N O P Q R S T U V W
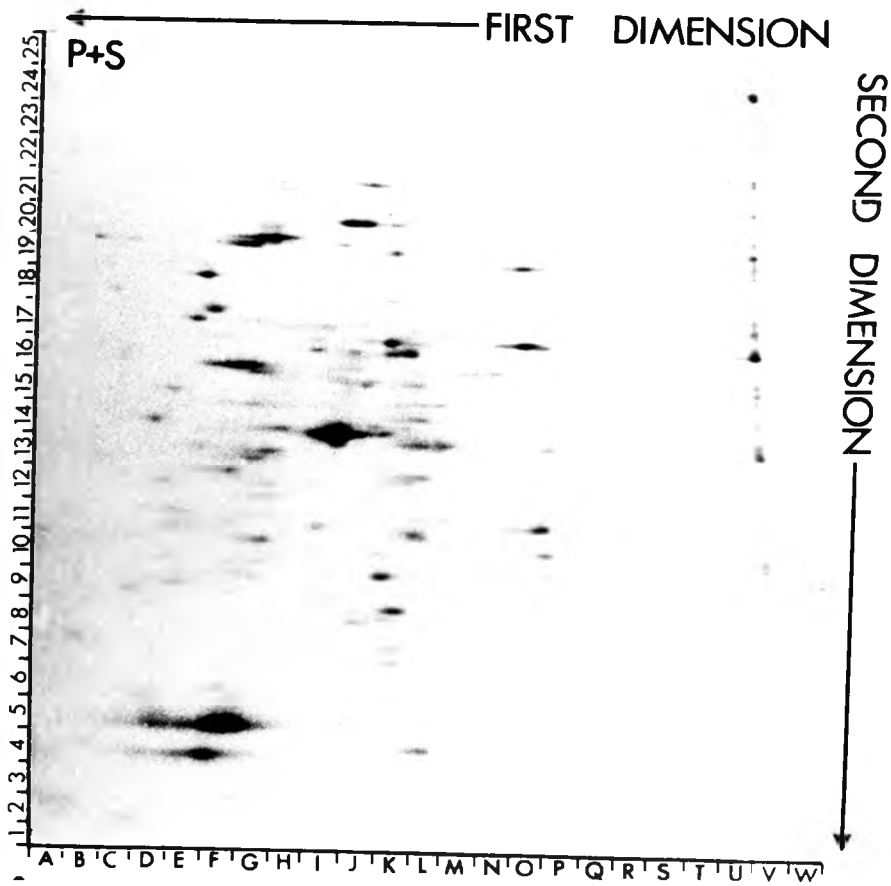
Plate 3.2

Two-dimensional gel autoradiograph of the S.P.F. of photosynthetically grown Rm 5 P +S cells labelled with ^{35}S methionine. Autoradiograph exposure time, 2 months.
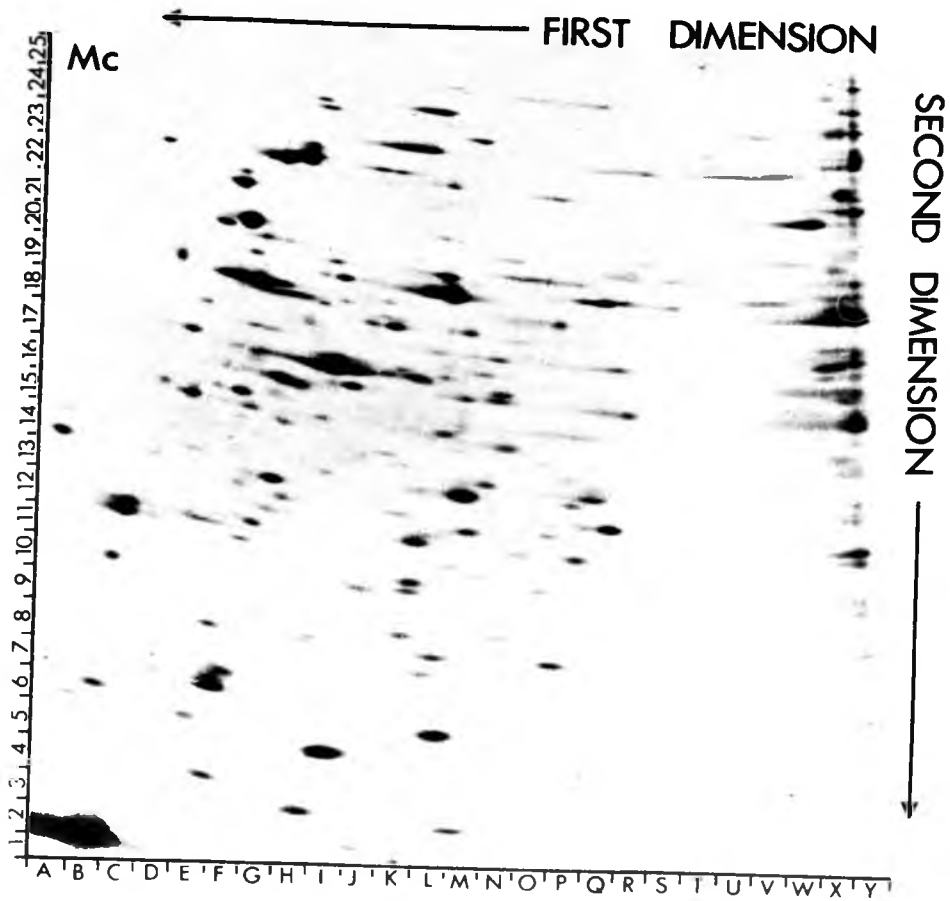
Plate 3.25

Two dimensional gel autoradiograph of the S.P.F. of photosynthetically grown <u>Rm5</u> 'normal Mc' cells labelled with ^{35}S methionine. Autoradiograph exposure time, 2 months.
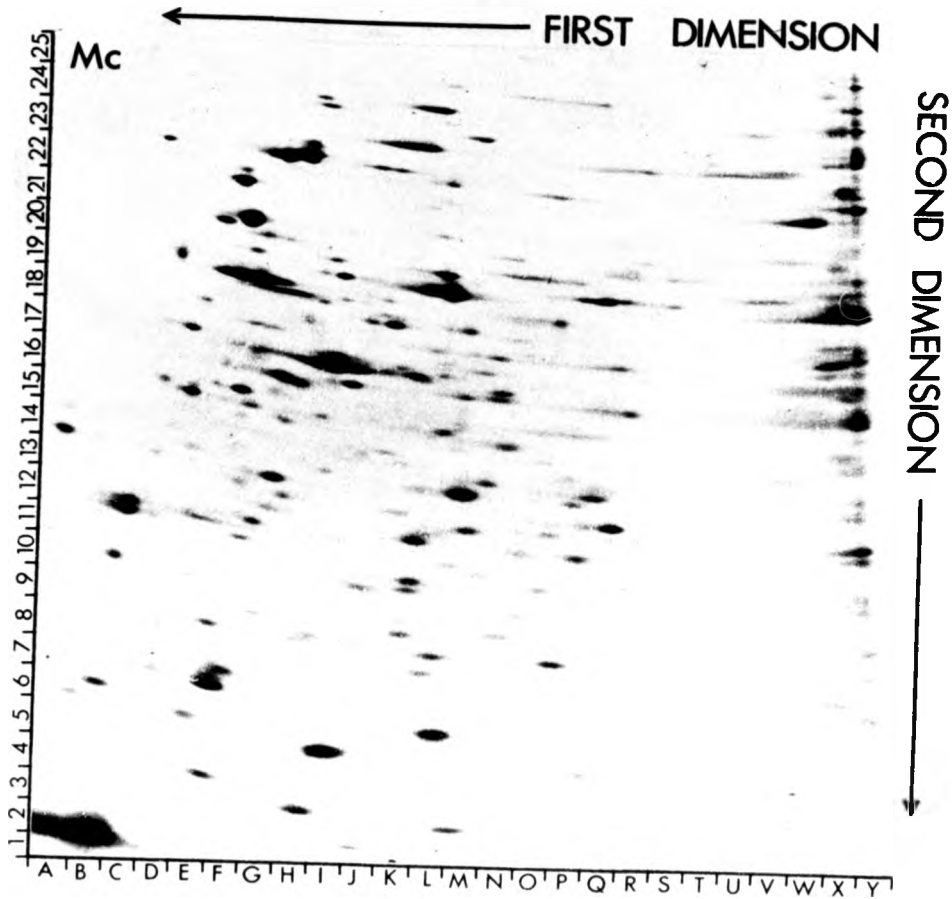
Plate 3.25

Two dimensional gel autoradiograph of the S.P.F. of photosynthetically grown Rm5 'normal Mc' cells labelled with ^{35}S methionine. Auto- radiograph exposure time, 2 months.
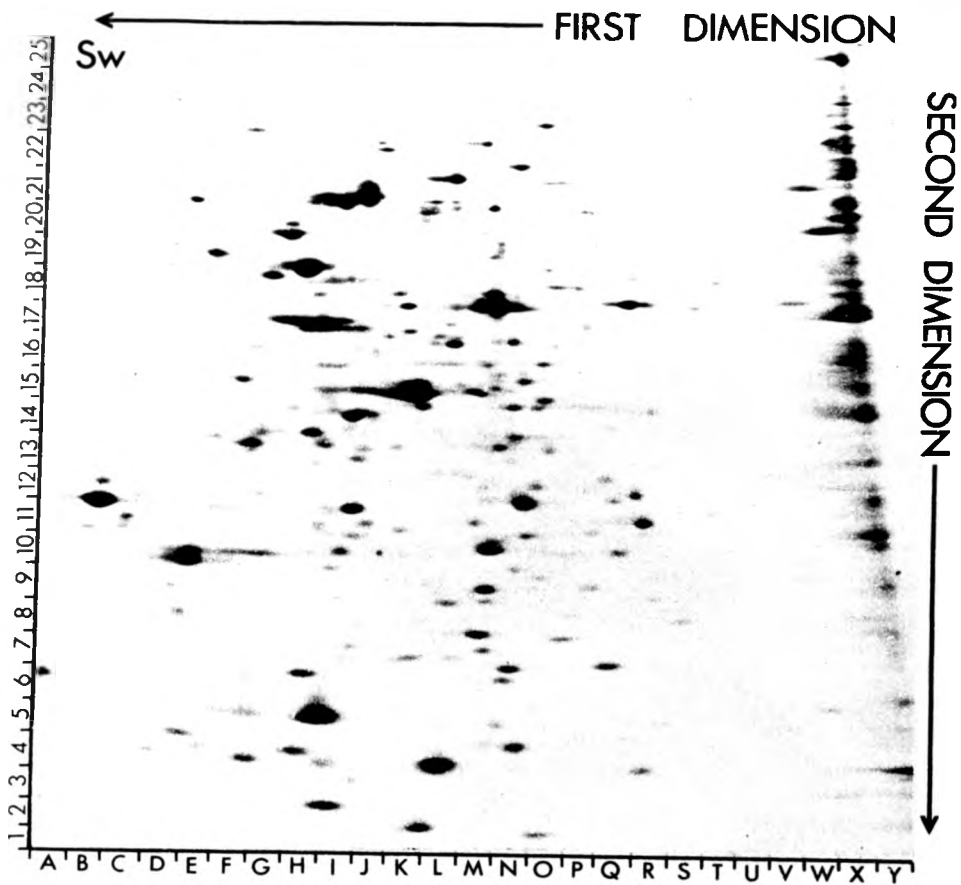
Plate 3.26

Two dimensional gel autoradiograph of the S.P.F. of photosynthetically
grown Rm5'normal Sw' cells labelled with ^{35}S methionine. Autoradiograph
exposure time, 2 months.
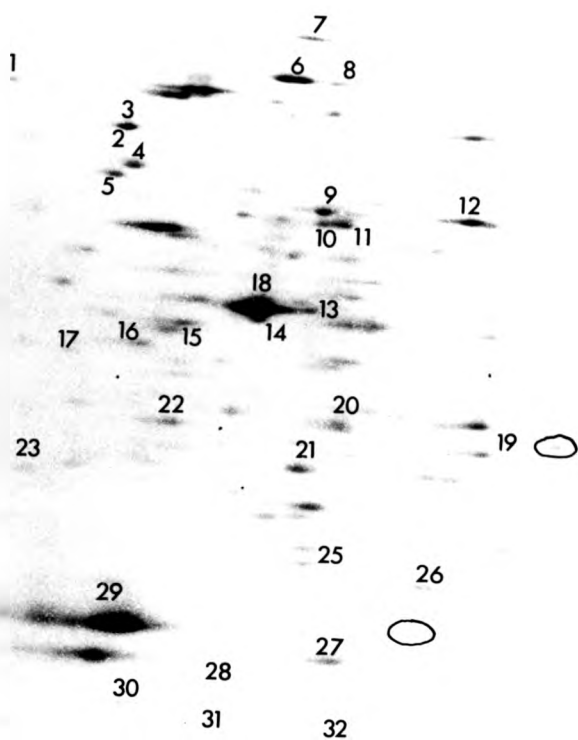
Plate 3.24A

Repeat of Plate 3.24. Polypeptides present on this separation (P + S) but absent from the Mc and Sw separations have been indicated by circles. Numbered 'spots' re present polypeptides common to all separations which can be used as references.

Plate 3.25A

Repeat of Plate 3.25. Polypeptides present on this separation (Mc) but absent from the Sw separation, have been indicated by circles.
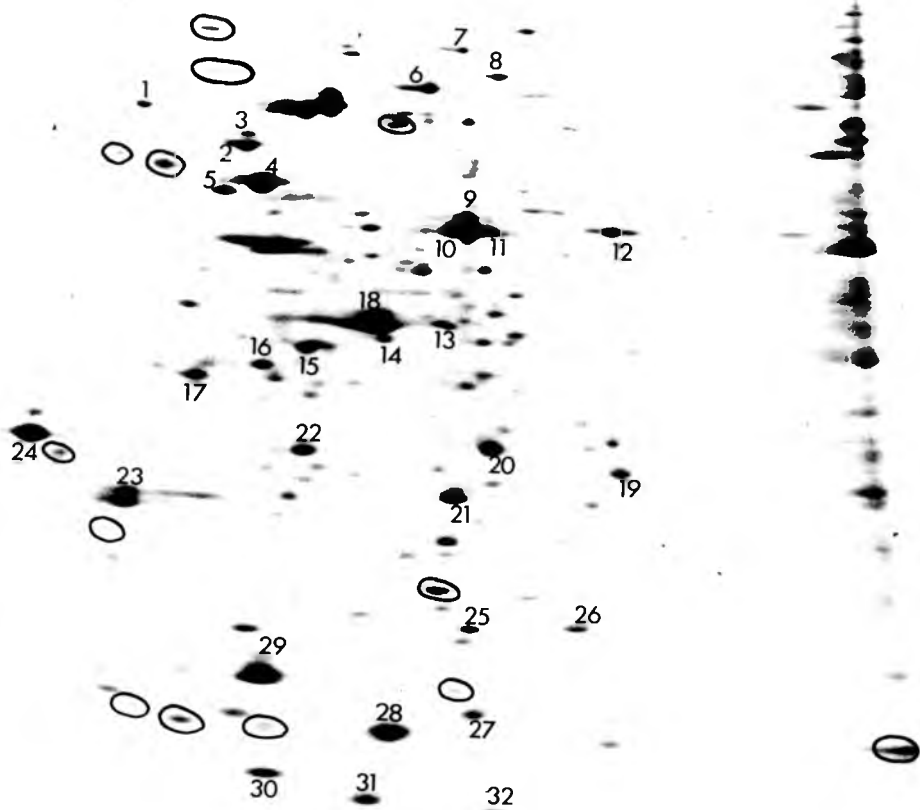
Plate 3.26A

Repeat of Plate 3.26. Polypeptides present on this separation (Sw) but absent from the Mc separation have been indicated by circles.
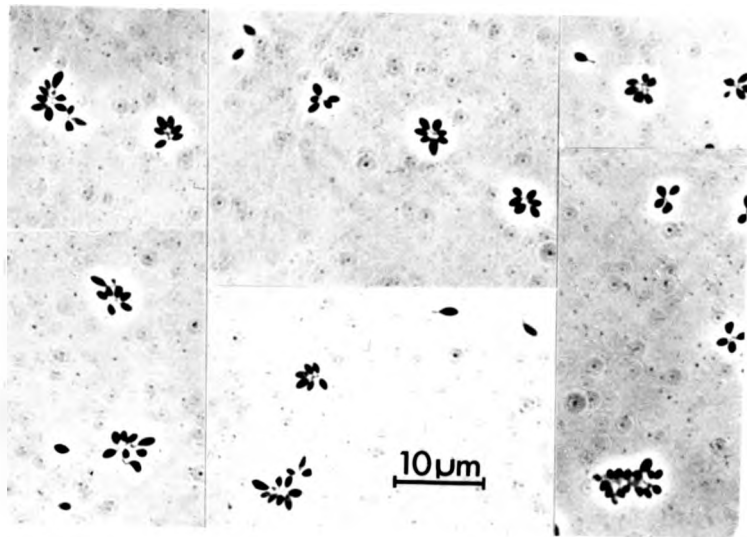
Plate 3. 27

Phase contrast photomicrographs showing the irregular clump-like
expression of a mutant Rm 5 isolate under conditions of low CO_2 tension.
(This isolate is expressed as P + S cells under conditions of high CO_2
tension).

HIGH CO_2 TENSION   LOW CO_2 TENSION
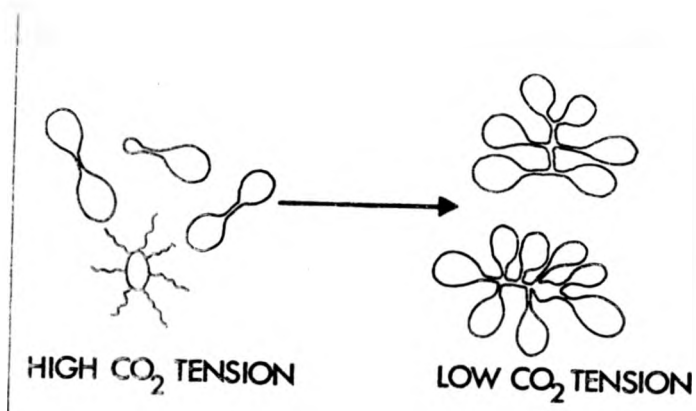
Fig. 3. 21

Diagrammatic representation of the cellular expression of the Rm 5
mutant (Plate 3. 27).

the 'normal' division processes. Had time permitted such a statement could have been verified by two dimensional gel electrophoresis analysis and the polypeptides involved been characterised. Missense mutations in phage T4 genes have been detected in this way (O'Farrell, 1975).

This technique offers great potential in the study of the protein changes occurring in the development of synchronized Rm 5 swarm cells through to mother cells, e.g. 'normal' Rm 5 swarm cells could be synchronized (glass wool column technique) and pulse labelled with ^{35}S methionine for 5 minutes at various stages during development. Two dimensional gel analysis of the soluble protein fractions would enable the synthesis of different polypeptides to be correlated with various stages of development. This technique also enables transcriptional control of cellular expression to be investigated in Rm 5. DNA dependent RNA polymerases from the different cell types (mother cells, swarm cells, exospores) can be purified and electrophoresed using this gel system and any modifications detected. RNA polymerases from the different cell types of other models of cellular differentiation have been compared by disc gel electrophoresis (Losick et al., 1970; Bendis and Shapiro, 1973), a technique far inferior with respect to resolution and sensitivity.

Similarly, control of differentiation in Rm 5 at the translational level can be studied using this technique. (Such experiments would be an extension of those by Fortnagel and Bergmann, (1973) who used disc gel electrophoresis to study ribosomal proteins of B. subtilis). Ribosomes from the different cell types of Rhodomicrobium can be purified, run on 2 dimensional gels and any differences in the ribosomal proteins detected.

The consequences of inhibitors of protein synthesis such as chloramphenicol, on the cell cycle can also be studied. Synchronized 'normal' Rm 5 swarm cells can be treated with different concentrations of inhibitor and the targets of inhibition pinpointed. A further extension would be the examination and characterization of the proteins involved in the initiation and completion of DNA replication. Selective and specific inhibitors of DNA synthesis would be particularly useful, and enable the developmental cycle of synchronous swarm cells to be dissociated from DNA replication. Hydroxyurea causes

cellular elongation but permits growth to continue, whereas nalidixic acid, mitomycin C and actinomycin D all inhibit growth and give gradients of development. The use of such inhibitors and two dimensional gel analysis would enable proteins dependent on DNA synthesis (e.g. 'termination' proteins, division proteins) to be distinguished from those synthesised irrespective of DNA replication or not.

(d)   Continuous culture studies

Apart from the initial isolation of the P + S cells using a chemostat and
the maintainance of a steady state culture reservoir, much of the early work
on these cells was performed using batch culture techniques (e.g. growth on
different carbon sources, at different light intensities, different pH's).
Under such conditions with a high number of variables, reproducibility of
results was difficult to achieve. Consequently the possibility of using a
chemostat for performing simple, in situ, physiological studies and
optimising growth conditions was investigated. Unless otherwise stated,
all the following continuous culture studies were performed using a 'Quickfit'
fermenter (working volume 650 ml) and routine growth medium (PYR/MAL
and 0.1 M phosphate buffer).

Variation of the incident light intensity

P + S cells from the culture reservoir were used as an inoculum and the
culture allowed to reach the mid-log phase of growth (absorbance$_{540} \sim 1.5$)
before continuous flow of fresh medium was commenced at 17 ml/h
$(D = 0.026 h^{-1})$. An incubation temperature of 30° C was maintained, and the
pH kept constant (pH 6.9 to 7.0) by the automatic addition of CO_2. The
first steady state was achieved using an incident light intensity of 3000 lux.
Illumination was then progressively reduced and further steady states
obtained. Because of the very slow dilution rate employed, the time allowed
for the culture to reach steady state was considerable and varied between
7 and 10 days. Culture absorbance at 540 nm (protein) and 890 nm (bacterio-
chlorophyll) was monitored together with cell volume (Table 3.5; Fig. 3.22;
Plate 3.28). Low overall culture densities were apparent. At very low
growth rates, energy consumption for maintenance purposes (purposes
other than growth) may become considerable in comparison with the energy
used for biosynthesis, and this can give rise to unusually low growth yields,
i.e. the functional status of the bacteria shifts to that of nutrient cycling
(Veldkamp, 1975). Such an explanation, however, was thought unlikely to
account for the low growth rate and culture density of the P + S cells since
growth limiting substrates were believed to be present in excess. Light
limitation of growth was apparent since an increase in culture density was
achieved by increasing the incident illumination. (For ideal behaviour,

Table 3.5

The effect of variation of the incident light intensity on the behaviour of <u>Rm 5</u> P+S cells in continuous culture ('Quickfit' chemostat, PYR/MAL medium + 0.1 M phosphate buffer, pH 6.8, 30°C. D = 0.026 h^{-1}).

	STEADY STATE				
Incident light intensity (lux)	Absorbance 540 nm	Absorbance 890 nm	Abs 890 /Abs 540	Peak channel number	Swarm cell volume (μm^3)
3000	1.52	0.75	0.49	15$\frac{1}{2}$	0.80
1500	1.52	0.76	0.50	17	0.84
900	1.23	0.71	0.58	17	0.84
600	1.20	0.79	0.66	18	0.86
300	1.16	0.88	0.76	21	0.94
150-200	1.15	0.97	0.84	25	1.04
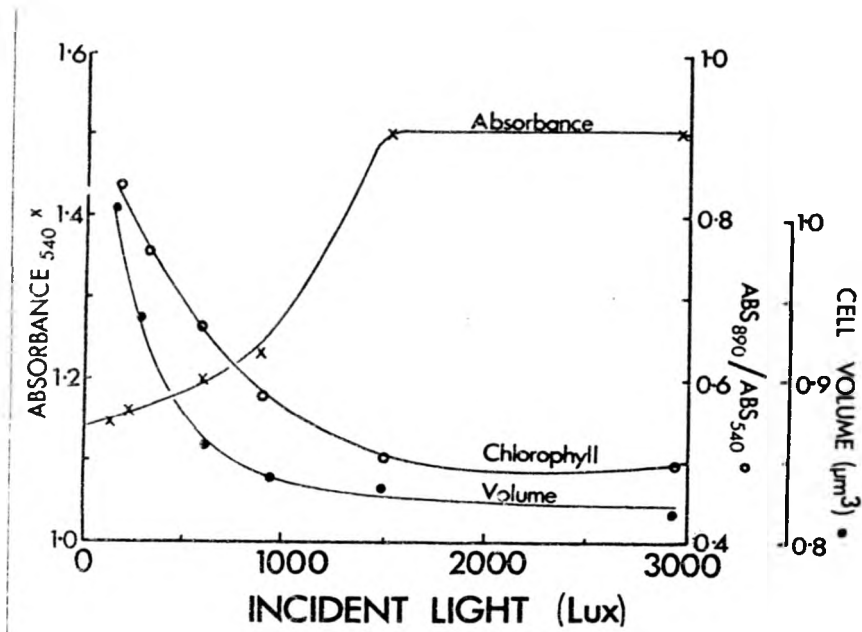
Fig. 3.22

The effect of incident light intensity variation on the characteristics of Rm 5
P + S cells in continuous culture, i.e. Table 3.5 expressed graphically.

(●) Swarm cell volume, μm^3. (Coulter channelyser).

(×) Culture density. (Absorbance 540 nm).

(o) Estimate of the membrane content of the cells .

Culture bacteriochlorophyll absorbance

Culture protein absorbance
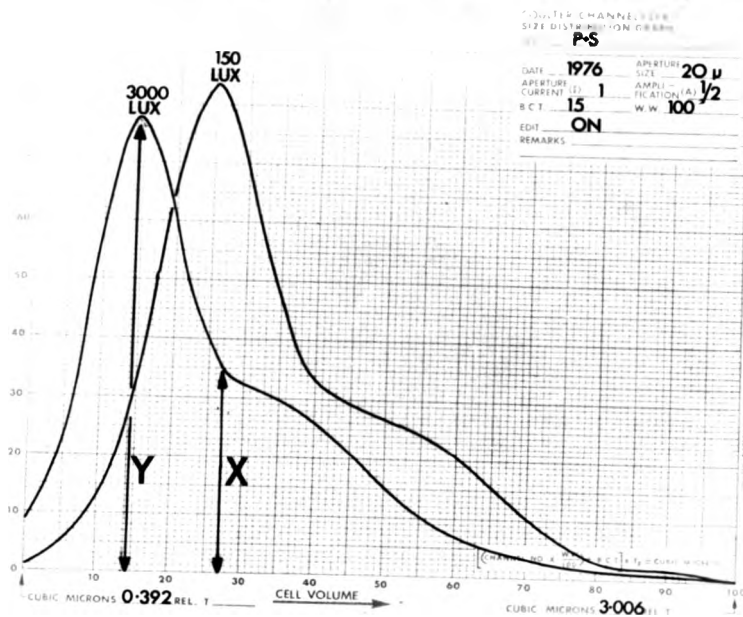
Plate 3.28

Coulter profiles of Rm 5 P + S cells from the high, and low, incident light intensity steady states.

Y represents the relative number of non-appendaged swarm cells

X represents the relative number of developing (appendaged) cells

The proportion of developing cells (X/Y) is greater at the higher light intensity steady state, i.e. there is less inhibition of swarm cell development.

biomass $\propto$ incident light intensity is expected if light is the limiting factor and everything else is in excess). Unfortunately, particle counts (Coulter counter) were not taken during this experiment but it can be surmised that cell number and biomass is increasing with light intensity up to at least 1500 lux. Cell volume is decreasing, therefore the increase in protein absorbance must be due to cell number increase. Above 1500 lux growth limitation is attributed to another factor. The observation that cell volume appeared to increase with decreasing illumination was at first surprising. A correlation between volume and bacteriochlorophyll content seemed to exist, and it appeared that the cells were increasing their volumes in order to physically accommodate the extensive photosynthetic membranes needed for growth at low light intensities. Examination of thin sections of cells by electron microscope revealed this to be so (Plates 3.29 and 3.30). Cells grown under low incident illumination had extensive photosynthetic membrane systems, whereas cells from the higher incident illuminations had a considerably reduced membrane lamellae system. As was found with R.rubrum (Holt and Marr, 1965) and R. sphaeroides (Sistrom, 1962b-Takemoto and Huang Kao , 1977) regulation of the photopigment content of Rm 5 P + S cells would appear to be achieved by varying the membrane content of the cells, i.e. one assumes a constant concentration of bacteriochlorophyll per unit length of membrane.

Some cells, especially those from the 1500 lux illumination steady state, were found to contain large numbers of polyhedral shaped cellular inclusions (Plate 3.31). The nature of these inclusions has not yet been determined, but they are similar in appearance to the polyglucose granules of Chlorobium thiosulphatophilum (Sirevag and Ormerod, 1977) and the carboxysomes and inclusion bodies of Thiobacillus intermedius and other Thiobacilli (Purokit et al., 1976; Shively et al., 1970). Similar polyhedral bodies have also been observed in Anabaena cylindrica (Codd and Stewart, 1976). D-Ribulose-1,5-Bisphosphate carboxylase is present in high concentrations in Rm 5 cells (3% (w/w) soluble proteins) (S. Taylor, this laboratory; personal communication) and one might speculate that under the conditions of very low dilution rate and fairly high light intensity, the enzyme is overproduced, and the cellular inclusions are, in fact, crystalline
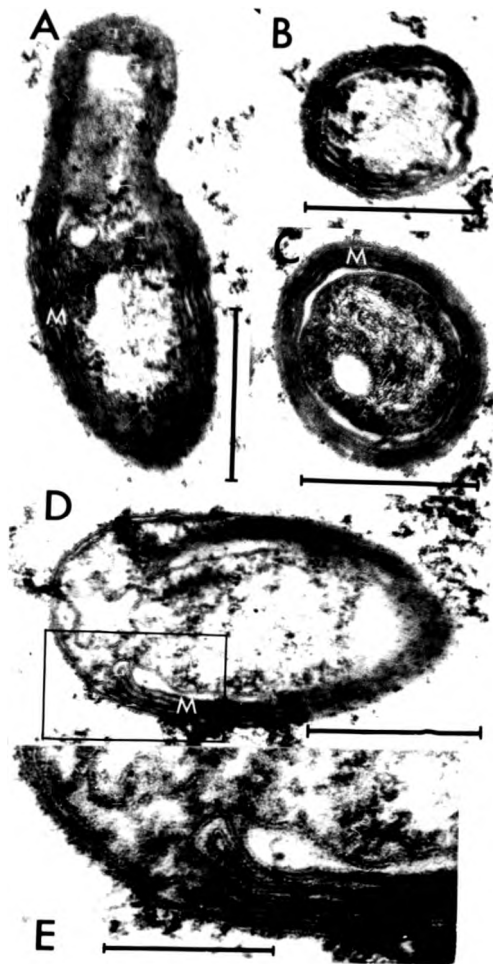
Plate 3.29   (A.B.C.D.   Bar = 0.5 μm
                      E.   Bar = 0.2 μm)

Ultrathin sections (Kellenberger fixation) of Rm 5 P + S cells grown in
continuous culture under low illumination (300 lux).

(A)(B)(C)(D)   Longitudinal and transverse sections showing the extensive
               peripheral membrane lamellar system.

(E)   Inset from longitudinal section D showing a membrane loop.
               (This grows along the length of the cell, infolds and returns).
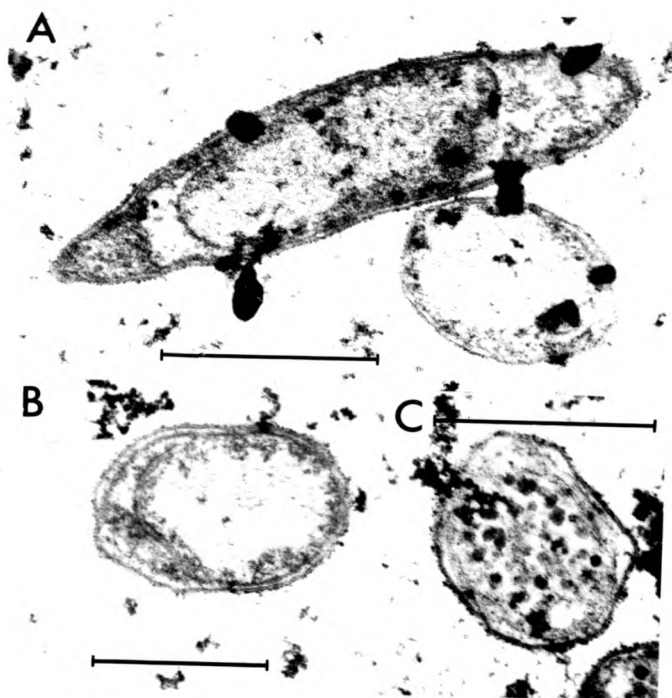
Plate 3.30   (Bars = 0.5 μm)

Ultrathin sections (Kellenberger fixation) of Rm 5 P + S cells grown in
continuous culture under high illumination (3000 lux).   Lamellar
membrane content considerably reduced.
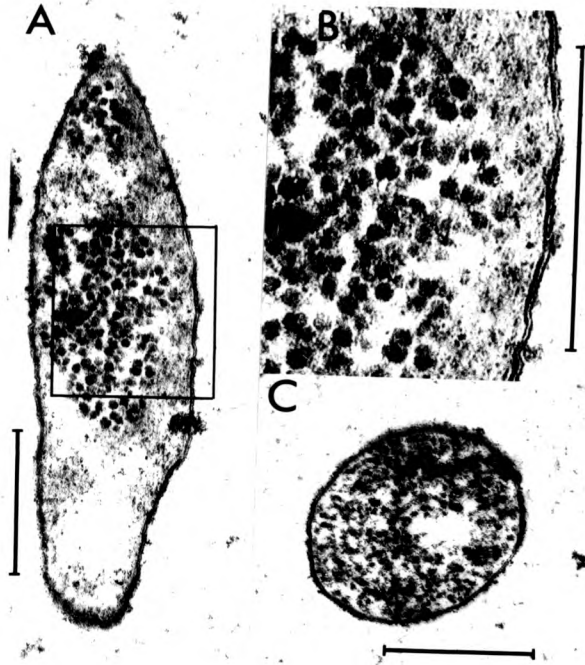
Plate 3.31   (Bars = 0.5 μm)

Ultrathin sections (Kellenberger fixation) of polyhedral inclusions apparent
in P +S cells growing in continuous culture with an incident light intensity
of 1500 lux.

(A)   Longitudinal section.

(B)   Transverse section.

(C)   Enlargement of the cell depicted in (A).

aggregates of D-Ribulose-1,5-Bisphosphate carboxylase. i.e.
carboxysomes.

The 'Coulter profiles' of the cells from the high and low illumination
steady states are very similar apart from the obvious volume shift
(Plate 3.28). Closer examination reveals that the height of the shoulder
of developing cells, relative to the height of the swarm cell peak (X/Y)
is greater in the cells growing at higher illumination, i.e. at the lower
illumination there are relatively fewer developing swarm cells. Inhibition
of development of immature swarm cells in 'normal' Rm 5 cultures at low
light intensity has also been observed (C.S.Dow, this laboratory; personal
communication).

Variation of dilution rate

Continuous culture work with Rm 5 P + S cells at 30° C proved difficult
and time consuming. The growth rate of Rm 5 is slow (doubling times of
4 h and above) and this necessitates the use of low dilution rates to avoid
washout of cells. Successful runs of 6 or 7 steady states were rare and
took up to 4 months to complete.

The object of this experiment was to find the maximum dilution rate
at which it was possible to grow P + S cells. An incubation temperature of
35° C was employed and an incident light intensity of 2000 lux. The
fermenter was inoculated, as before, with log phase P + S cells from the
steady state culture reservoir, and allowed to reach the mid-exponential
growth phase before continuous flow of fresh medium (PYR/MAL and 0.1 $\underline{M}$
phosphate buffer, pH 6.8) was commenced at 65 ml/h . The pH was kept
constant at 7.0 using CO_2 addition as a pH control. Samples were removed
at regular intervals (cell count, cell volume, absorbance 540 nm) until a
steady state was obtained. The dilution rate was then progressively
increased and further steady states reached (Table 3.6; Fig. 3.23).

Under the conditions employed, it was possible to grow the cells at
relatively high dilution rates (0.161h)[1] with doubling times of up to 4.3 h .
The growth rates of the cells was greatly improved by increasing the
incubation temperature to 35° C. Surprisingly, however, an increase in
dilution rate did not significantly alter the cell volume. The 'Coulter
profile' of cells grown at a dilution rate of 0.10 h^{-1} was identical, with respect
to both curve shape and swarm cell peak position, to the 'Coulter profile' of
the cells growing at the higher dilution rate (0.16 h^{-1})(Plate 3.32).Unlike the

Table 3.6

The effect of variation of the dilution rate on the behaviour of Rm 5
P + S cells in continuous culture. ('Quickfit' chemostat, PYR/MAL medium
+ 0.1 M phosphate buffer, pH 6.8, 35°C, 2500 lux).

STEADY STATE

Dilution rate (h^{-1})	Absorbance 540 nm	Particle count per ml. (Coulter counter)	Mean swarm cell volume (μm^3)	Mean cell doubling time (h.)
0.1	1.24	5.75×10^8	0.89	6.9
0.123	0.80	4.05×10^8	0.94	5.6
0.129	0.92	3.58×10^8	0.96	5.4
0.14	0.74	3.07×10^8	0.94	4.9
0.161	0.48	1.89×10^8	0.96	4.3

Fig. 3.23.

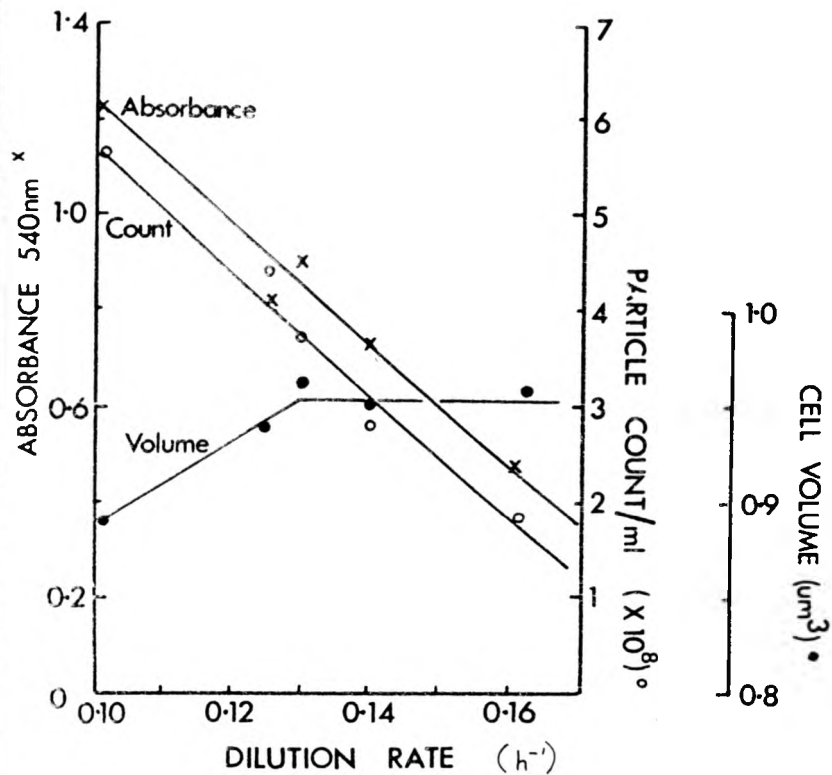The effect of dilution rate variation on the characteristics of Rm 5 P + S cells in continuous culture i.e. Table 3.6 expressed graphically.

(●) Swarm cell volume (μm^3)(Coulter channel yser).

(×) Culture density. Absorbance 540 nm.

(○) Culture density. Particle count (Coulter counter).
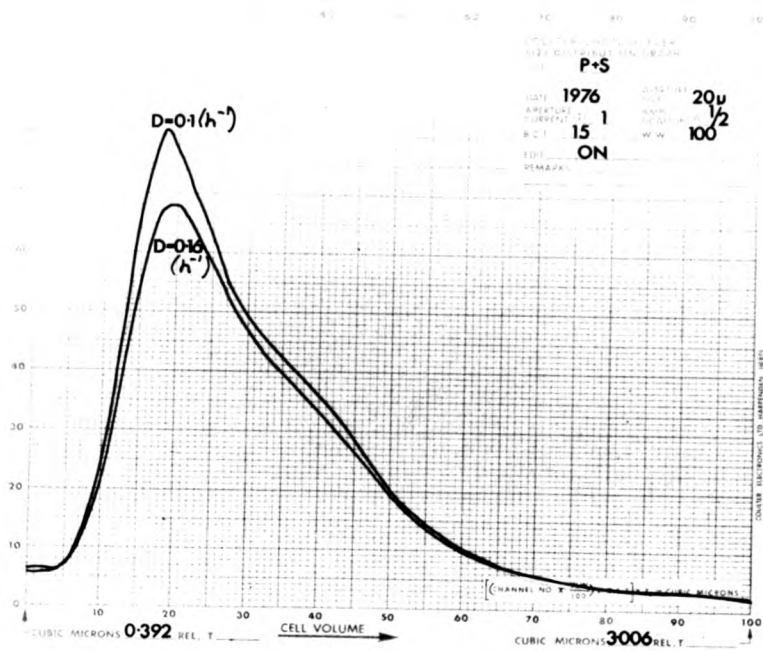
Plate 3.32

'Coulter profiles' of Rm5 P + S cells from the high and low dilution rate steady states.

behaviour of other cells in continuous culture (e.g. Bacillus megaterium,
Herbert, 1958, 1961; Salmonella typhimurium, Schaecter et al., 1958;
Arthrobacter spp. Luscombe and Gray, 1971), the cell size and
morphology of Rm 5 did not appear to be appreciably affected by growth
rate. A marginal increase in volume was apparent between the dilution
rates of 0.10 and 0.13h^{-1} but above this there was no detectable change.
One hypothesis is that any volume increase due to a higher growth rate may
well be masked or balanced by the converse effect of the change in light
intensity on the cell size at the respective steady states. [At high dilution
rates, results indicate that cell number and culture absorbance decreases.
This in turn leads to an increase in the amount of light energy available
per cell, compensated for by a subsequent reduction in the photosynthetic
membrane content. Correlation between cell volume and photosynthetic
membrane content was suggested by results of the previous experiment
(steady state analysis at different incident light intensities)]. Such an
explanation, however, seems unlikely in view of the fact that the experiment
was carried out at a relatively high incident light intensity (2000 lux) and
examination of ultrathin sections at the different steady states revealed
little difference in the membrane content of the cells. It may be worthwhile
repeating this experiment using a turbidostat. Nevertheless, although the
conditions had very little effect on cell volume, at the high dilution rates
shorter cell filament lengths were apparent. In the prosthecate
Hyphomicrobium (which bears a close resemblance morphologically to
Rm 5 P +S cells) a similar effect of growth rate on filament length was
reported by Harder (1974). Growth of P + S cells at high dilution rates also
led to the appearance of large numbers of 'triplet' configurations in which
the mother cells were producing new daughter cells before separation of
the previous one had been completed.

At the higher dilution rates, growth of the cells on the walls of the
fermenter vessel caused problems. This was removed every 12 h by
scraping the sides. The general effects of wall growth have been discussed
by Topiwala and Hamer (1971) and by Solomons (1972), and when it occurs
a true steady state cannot be obtained. Obviously the light intensity inside
the vessel is affected to an unknown extent. Culture characteristics are

behaviour of other cells in continuous culture (e.g. Bacillus megaterium, Herbert, 1958, 1961; Salmonella typhimurium, Schaecter et al., 1958; Arthrobacter spp. Luscombe and Gray, 1971), the cell size and morphology of Rm 5 did not appear to be appreciably affected by growth rate. A marginal increase in volume was apparent between the dilution rates of 0.10 and 0.13h^{-1} but above this there was no detectable change. One hypothesis is that any volume increase due to a higher growth rate may well be masked or balanced by the converse effect of the change in light intensity on the cell size at the respective steady states. [At high dilution rates, results indicate that cell number and culture absorbance decreases. This in turn leads to an increase in the amount of light energy available per cell, compensated for by a subsequent reduction in the photosynthetic membrane content. Correlation between cell volume and photosynthetic membrane content was suggested by results of the previous experiment (steady state analysis at different incident light intensities)]. Such an explanation, however, seems unlikely in view of the fact that the experiment was carried out at a relatively high incident light intensity (2000 lux) and examination of ultrathin sections at the different steady states revealed little difference in the membrane content of the cells. It may be worthwhile repeating this experiment using a turbidostat. Nevertheless, although the conditions had very little effect on cell volume, at the high dilution rates shorter cell filament lengths were apparent. In the prosthecate Hyphomicrobium (which bears a close resemblance morphologically to Rm 5 P +S cells) a similar effect of growth rate on filament length was reported by Harder (1974). Growth of P + S cells at high dilution rates also led to the appearance of large numbers of 'triplet' configurations in which the mother cells were producing new daughter cells before separation of the previous one had been completed.

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This in turn leads to an increase in the amount of light energy available
per cell, compensated for by a subsequent reduction in the photosynthetic
membrane content. Correlation between cell volume and photosynthetic
membrane content was suggested by results of the previous experiment
(steady state analysis at different incident light intensities)]. Such an
explanation, however, seems unlikely in view of the fact that the experiment
was carried out at a relatively high incident light intensity (2000 lux) and
examination of ultrathin sections at the different steady states revealed
little difference in the membrane content of the cells. It may be worthwhile
repeating this experiment using a turbidostat. Nevertheless, although the
conditions had very little effect on cell volume, at the high dilution rates
shorter cell filament lengths were apparent. In the prosthecate
Hyphomicrobium (which bears a close resemblance morphologically to
Rm 5 P +S cells) a similar effect of growth rate on filament length was
reported by Harder (1974). Growth of P + S cells at high dilution rates also
led to the appearance of large numbers of 'triplet' configurations in which
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fermenter vessel caused problems. This was removed every 12 h by
scraping the sides. The general effects of wall growth have been discussed
by Topiwala and Hamer (1971) and by Solomons (1972), and when it occurs
a true steady state cannot be obtained. Obviously the light intensity inside
the vessel is affected to an unknown extent. Culture characteristics are

also influenced since the wall growth can act as a captive population in the fermenter, continuously discharging its progeny ('normal' swarm cells) into the culture suspension and thus giving rise to a 'mixed' culture. Drastic changes in the characteristics of mixed chemostat cultures of Hyphomicrobium by wall growth have been described by Wilkinson and Hamer (1974).

It is evident that at the high dilution rates the P + S cells lose stability and change back to the normal form of cellular expression, since the presence of multicellular arrays can be observed under the light microscope. It is possible that, at high dilution rates, the pH is not controlled by the CO_2, but being held by the buffer. A reduction in the amount of CO_2 being added externally would be inevitable with a concomitant drop in the CO_2 tension of the culture medium. Conditions then become favourable for the growth of 'normal' heterogeneous cells. When wall growth, and the number of 'normal' cells, eventually became excessive, the run was abandoned.

Variation of CO_2 tension on the stability of P + S cells at high dilution rates

As an extension to the previous experiments, P + S cells were grown in the chemostat at a high dilution rate and the effect of increasing the CO_2 tension on the culture stability monitored. An incubation temperature of 35° C and an incident light intensity of 2000 lux were employed. Cells from the steady state culture reservoir were used as inoculum and allowed to grow to the mid-exponential phase before the medium flow (PYR/MAL and 0.1 $\underline{M}$ phosphate buffer, pH 6.8) was commenced (72.7 ml/h , D = 0.112 h^{-1}). CO_2 was used to control the pH and a steady state was obtained. Cell growth on the walls of the fermenter vessel soon became evident and was attributed to microcolonies and chains of cells (multicellular arrays). The automatic pH controller was then disconnected and CO_2 pumped continuously into the culture medium. A pH drop (6.8 to 6.45) was incurred as a result, but a new steady state was eventually reached. The (gas flow) rate of addition of CO_2 to the culture was then progressively increased and further 'steady' states at higher CO_2 tensions (and lower pH values) were attempted. Cell growth on the sides of the fermenter vessel was removed every 12 h . Table 3.7 shows the results obtained. An increase in the CO_2 tension

Steady state	pH	Absorbance 540 nm	Particle count/ml (Coulter counter)	Mean swarm cell volume (μm^3)	Wall growth
1	6.8	1.20	4.9×10^8	0.915	+
2	6.45	1.06	4.6×10^8	0.980	+
(3)	6.00	0.60	3.0×10^8	1.098	+
(4)	5.85	0.20	1.0×10^8	1.18	+

Table 3.7

The effect of variation of the CO_2 tension (and pH) on the characteristics of Rm 5 P+S cells growing in continuous culture at a relatively high dilution rate. ('Quickfit' chemostat, PYR/MAL medium + 0.1 M phosphate buffer, pH 6.8, 35° C, 2000 lux, D = 0.112 h^{-1}).
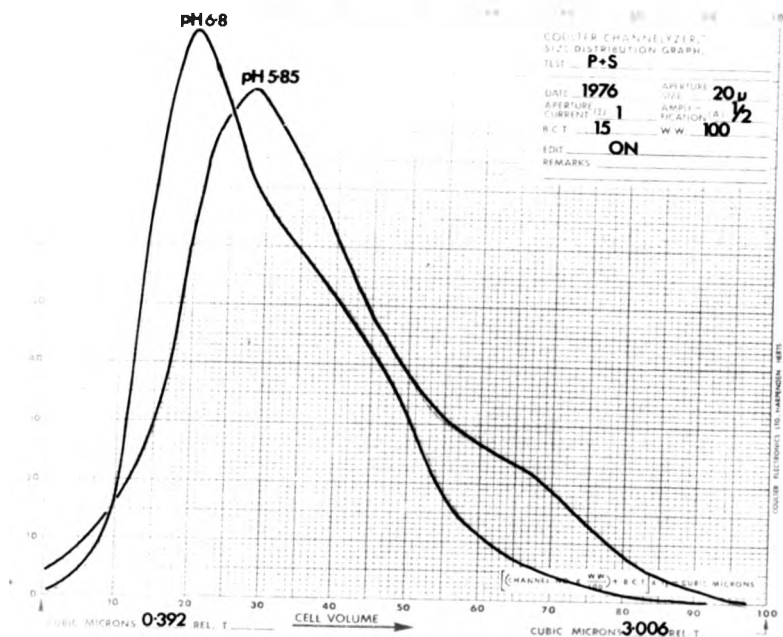

Plate 3.33

'Coulter profiles' of the cells from steady state (1) and steady state (4). (Table 3.7).

appeared to have little effect on preventing the loss of stability of the
P + S cells at high dilution rates. Instability of growth of P + S cells at
30° C in a chemostat with CO_2 pH control, however, was never observed.
The solubility of CO_2 is reduced at higher temperatures and a significant
reduction in the soluble CO_2 tension may well contribute to the instability
of the P + S cellular expression at 35° C. Alternatively the P + S cells
may be temperature sensitive, although this seems unlikely, since under
batch conditions P + S cells will grow satisfactorily at incubation
temperatures up to 40° C.

At the lower pH values, P + S cell growth was considerably limited and
there was a significant drop in the culture absorbance and Coulter count.
In addition there was an increase in the cell volume with a characteristic
change in the 'Coulter profile' (Plate 3.33). Under the light microscope
the cells were elongated and distorted, suggesting that the cell division
processes were affected. This resulted in eventual culture washout.

Effect of changing from CO_2 to acid pH control.

This experiment is described in section 3e.

Continuous culture of 'normal' heterogeneous Rm 5

Only by using an L.H.E. 1/1000 laboratory fermenter was it possible to
grow a 'normal' culture of Rm 5 continuously, and maintain a steady state.
An incubation temperature of 30° C and an incident light intensity of 4000
lux were employed. The pH (7.55) was controlled using 0.1 M HCl and
agitation of the culture was relatively slow (stirrer rate, 200 r.p.m.).
An exponential culture was used as inoculum and allowed to grow to the
mid-exponential phase of growth (absorbance$_{540} \sim 1.5$) before the medium
(PYR/MAL and 0.1 M phosphate buffer, pH 6.8) flow was started (62 ml/h
$D = 0.031 h^{-1}$). This flow rate was later increased to 93 ml/h when the
culture absorbance reached approximately 1.9. A drop in culture density
occurred and a steady state was reached.

Steady state $(30^o$ C, 200 r.p.m., 4000 lux)

Culture absorbance 540 nm	1.4
Swarmer absorbance 540 nm	0.04
pH	7.55

The steady state culture was composed almost entirely of multicellular arrays (microcolonies and chains of cells) although low numbers of motile swarm cells and the occasional exospore were also present. Growth of cells on the sides of the fermenter vessel was also evident but this was removed by intermittently increasing the stirring rate to its maximum value. High agitation rates for considerable lengths of time were deleterious to cell growth resulting in breakage of prostheca, cell lysis and culture washout.

(e) Simplified cell cycle.   Mutation or a response to an environmental stimulus ?

Work has been presented on the morphology and physiology of the P + S cellular expression of Rm 5 in batch and continuous culture. Perhaps the most significant question to ask concerns the origin of this simplified cell cycle.   Is it the expression of another cell type of Rm 5, produced as a response to environmental conditions, and if so what is the stimulus ? Aleternatively are P + S cells mutants selected out by the chemostat ?

15 litre batch fermenter studies

The following section (3f) of this thesis is confined to the behaviour of Rm 5 under different environmental conditions in a 15 litre batch fermenter. Initially, P + S cells were observed and isolated in a chemostat but detailed work has also revealed the presence of such cells, albeit in very low numbers, under 'normal' batch conditions. Consequently it is necessary to partially detach one of a series of experiments and discuss the results separately.

A 'normal' culture of Rm 5 was grown in the 15 litre batch fermenter on routine medium (PYR/MAL and 0.1 M phosphate buffer, pH 6.8) at 30° C with an incident light intensity of 9000 lux. The CO_2 tension in the culture was monitored using the silicone coil/katherometer technique. Samples were removed at regular intervals for the measurement of culture absorbance, and swarm cells were separated (glass wool column technique) serially diluted, and plated out on to solid medium. The plates were incubated anaerobically in the light, and the colonies scored for type (rough or smooth) and number (Fig. 3.24).

A typical lag phase, exponential phase, stationary phase growth curve was observed for the culture. However, an initial drop in the viable swarm cell number was evident, and probably a consequence of the change in environmental conditions (light, CO_2 tension) experienced by the log phase inoculum being diluted into 15 litres of fresh medium. Swarm cells were produced throughout exponential growth to a maximum of 6×10^6/ml, before a slight reduction in the number occurred prior to the onset of the stationary phase. No smooth swarm cell colonies were detected until after
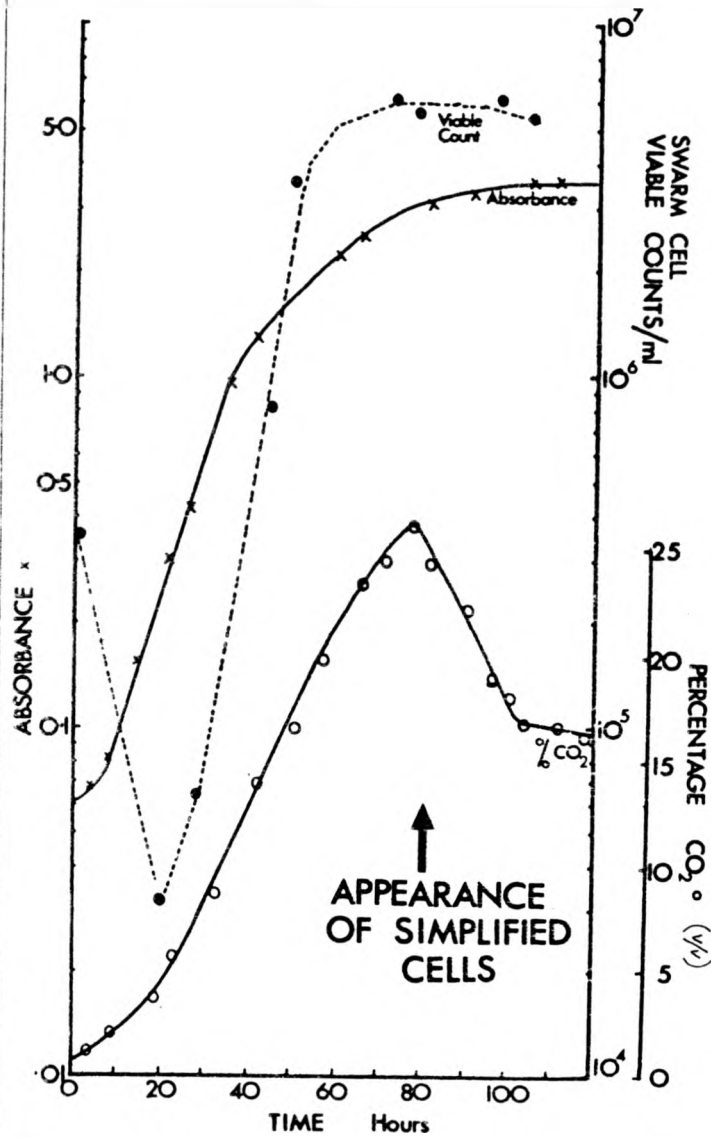
Fig. 3.24

Some characteristics of 'normal' heterogeneous Rm 5 cells grown photo-
synthetically in a 15 litre batch fermenter (PYR/MAL medium + 0.1 M
phosphate buffer, pH 6.8, 30°C, 9000 lux, stirrer rate 100 r.p.m.).

(X)   Culture density . (Absorbance 540 nm).

(o)   %(v/v) CO_2 in suspension (Silicone coil/katherometer technique).

(•)   Swarm cell viable count. (Swarm cells separated by the glass wool
        column technique, plated out, and incubated in an 'anaerobic bag'.

about 75 h of culture growth when the culture density was relatively high (absorbance$_{540}$ ~ 3.0). 1 - 2% of the swarm cells produced then gave rise to smooth colonies, characteristic of cells of the simplified cycle. The production of this colony type coincided with the maximum CO_2 tension of the culture medium, which may indicate that CO_2 is involved as the stimulus for expression of the simplified cell cycle. Moreover, the involvement of CO_2 as a probable trigger for differentiation in other organisms has been widely implicated (Lones and Peacock, 1960; Bartnicki-Garcia and Nickerson, 1962a;Roberts, 1964; Elmer and Nickerson, 1970).

Chemostat studies. Acid or CO_2-pH control

Rm 5 P +S cells were grown in a 'Quickfit' chemostat under relatively high CO_2 tension (gaseous CO_2 as pH control agent) which was then considerably reduced by changing over to acid pH control. The cells were grown at $30°$ C with an incident light intensity of 2000 lux. Anaerobiosis was maintained by bubbling oxygen-free nitrogen through the culture at a flow rate of 25 ml/min.

A normal culture of Rm 5 was used as inoculum and P + S cells were selected out by the batch run technique (section 3a) using CO_2 to control pH until a steady state was obtained (dilution rate = $0.0184h^{-1}$). Control of pH was then changed from CO_2 to acid (0.1 M HCl) and the behaviour of the cells closely followed. Culture composition (% P + S) was evaluated using the syringe technique. Fig. 3.25 shows the results obtained.

Reduction of the culture CO_2 tension had an adverse effect on the cells and resulted in a drop in the culture density. After 12 days the culture density was nearly half its steady state value, and its composition was significantly altered. Growth of cells on the walls of the fermenter vessel was evident, and a large number of 'normal' cells (multicellular arrays) could be observed under the light microscope. This was reflected by a significant drop in the measured percentage of P + S cells in the culture. Changing the pH control back to carbon dioxide had little immediate effect on the cellular expression. However, after approximately 4 days of increased CO_2 tension, the culture absorbance began to increase. Disappearance of 'normal' cells was evident, and the percentage of P + S
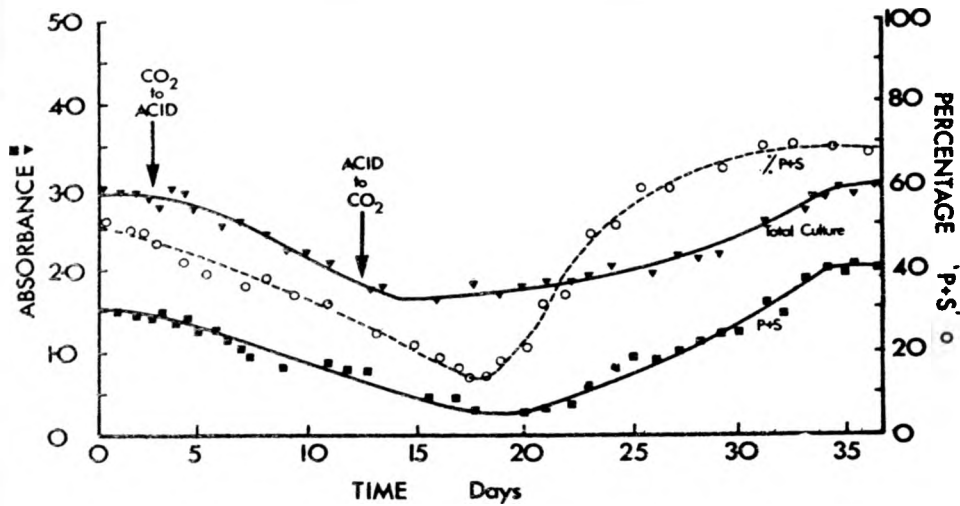
Fig. 3.25

The effect of regulating the CO_2 tension (by changing from CO_2 to acid pH control and _vice versa_) on the behaviour and expression of Rm 5 cells growing photosynthetically in continuous culture at low dilution rate. ('Quickfit' chemostat, PYR/MAL medium + 0.1 M phosphate buffer, pH 6.8, 30° C, 2000 lux. $D = 0.018 h^{-1}$).

($\blacktriangledown$) Total culture density. Absorbance 540 nm.

($\blacksquare$) P + S density. Absorbance 540 nm. P + S cells were separated from the multicellular arrays using 50 ml syringes packed with glass wool (Section 2).

($\circ$) % P + S cells (i.e. $\dfrac{\text{Absorbance 540 nm P + S}}{\text{Absorbance 540 nm total culture}} \times 100$)

cells increased. Recovery of the P + S cells occurred and a steady state culture composed of simplified cells was obtained. It is, therefore, apparent that CO_2 is involved in maintaining expression of the simplified cell cycle.

Environmental influence on swarm cell expression

Here the object was to grow 'normal' Rm 5 swarm cells in batch culture under different environmental conditions to identify those, if any, which influenced the expression of the simplified cell cycle.

'Normal' heterogeneous Rm 5 was cultured in a 15 litre batch fermenter (30^oC, 9000 lux incident illumination) on routine medium and the swarm cells separated by the glass wool column technique. These were then inoculated (20 ml) into duplicate 250 ml B19 shake flasks containing 100 ml of PYR/MAL growth medium and 0.1 M phosphate buffer. Growth medium of different pH values (6.0 to 8.0) was employed and also medium supplemented with different concentrations of sodium bicarbonate (0.002 % (w/v) to 0.02 % (w/v)). Incident light intensity was also used as a parameter. All flasks were incubated at 30° C. Samples were removed after 24 h incubation for microscopic examination and plating out on to solid medium for colony type analysis (Table 3.8). None of the conditions employed significantly increased the frequency of smooth colonies (P + S). The control flask which contained 120 ml of swarm cells in original eluent medium had a frequency of smooth colony occurrence of about 0.18 %. Only three of the altered parameters gave smooth colony frequencies higher than this, but there was no evidence to suggest that, individually, either light intensity, pH or CO_2 tension influenced P + S cell production. Simple environmental control appears unlikely and a combination of environmental influences may be responsible for the expression of the simplified cell cycle of Rm 5.

Perhaps the most striking observation of these experiments concerned swarm cells 'growing' aerobically in the dark. Motility was retained for at least 48 h and development was completely arrested, which gives further support to the fact that swarm cell development is inhibited by low illumination (C.S. Dow, personal communication).

Table 3.8

The effect of different environmental conditions (light, pH, CO_2 tension) on the expression of 'normal' heterogeneous Rm 5 swarm cells. (Culture samples (batch) were removed after 24 h incubation at 30° C and examined microscopically. Samples were also plated out and incubated anaerobically for colony type analysis).

Environmental Conditions		Microscopic Examinations	Rough colonies stored	Smooth colonies stored	% simplified cells
Control		'Normal' development	1103	1	0.18
(ph 7.0)	200	'Normal' development	1436	0	−
Incident light intensity (Lux)	800	'Normal' development	1208	1	0.08
	3000	'Normal' development	2392	2	0.08
	Aer dark	Little growth. Swarm cells motile	500	0	−
(2000 lux)	6.0	'Normal' development; good growth	2811	4	0.14
	6.4	'Normal' development; good growth	2095	3	0.14
Initial medium pH	6.8	'Normal development; good growth	800	2	0.25
	7.2	'Normal' development; good growth	1300	4	0.30
	7.6	'Normal' development; good growth	1165	0	−
	8.0	'Normal' development; good growth	1000	1	0.1
(pH. 7.0 2000 lux)	0.002	'Normal development; good growth	1244	1	0.08
	0.005	'Normal' development ;good growth	975	1	0.10
Bicarbonate conc. %(w/v)	0.007	'Normal' development; good growth	647	0	−
	0.01	'Normal' development; very good growth	804	0	−
	0.02	'Normal' development; poor growth	870	3	0.35

Fluctuation test

An extension of the system used by Luria and Delbrück (1943) was applied to Rm 5 to determine whether P + S cells arise by mutation or as a response to environmental stimuli.

A stationary phase culture of 'normal' heterogeneous Rm 5 was used to inoculate 20 independent subcultures which were then grown under identical conditions. 500 ml bottles containing routine growth medium (500 ml PYR/MAL and 0.1 M phosphate buffer) were used as culture vessels and incubation was at 30° C with incident illumination of 1000 lux. Samples from each culture were removed during very early and very late exponential growth and rapid separation of the swarm cells was effected by passage through a syringe packed with glass wool. The individual swarm cell fractions were then serially diluted and plated out on to solid medium. The plates were incubated in an 'anaerobic bag' and the colonies scored for type and number.

The mutation hypothesis would lead to the following prediction:- although the independent cultures are the same with respect to total size of population, some may have experienced a mutation at an early generation and thus yield a high number of P + S colonies (smooth) by the late exponential phase, whilst with others a mutation may have occurred during very late exponential phase, so resulting in only a few P + S colonies, i.e. the independent cultures would show a wide fluctuation in their content of P + S cells.

However, if P + S cells are a phenotypic expression of Rm 5 under environmental control, each culture should produce approximately the same number or proportion of smooth colonies.

The results obtained, however, was disappointing, inadequate and inconclusive (Table 3.9). The mutation theory may possibly have been favoured. Less than half the cultures gave rise to smooth colonies in the late exponential phase samples, and if environmental control was operating all would have been expected to give rise to some P + S cells. A fluctuation in the number and percentage of smooth colonies is apparent in the late exponential phase samples and this, taken on its own, would give support to the mutation theory. However, 9 of the early exponential phase samples gave rise to smooth colonies and these cultures would be expected to give rise to large clones of P + S cells and significantly higher percentages of smooth colonies by late exponential phase. This, however, was not the case.

Table 3.9   Results of 'fluctuation test'

Bottle number	Early exponential phase sample (24h)			Late exponential phase sample (167 h)		
	Rough colonies	Smooth colonies	%simplified cells	Rough colonies	Smooth colonies	% simplified cells
1	880	0	0	3058	2	0.06
2	988	0	0	1690	3	0.17
3	1320	3	0.2	2932	1	0.03
4	950	0	0	1215	0	0
5	1000	0	0	492	0	0
6	622	0	0	194	0	0
7	301	0	0	4560	0	0
8	500	0	0	2388	5	0.21
9	888	0	0	2400	0	0
10	2456	1	0.04	1062	0	0
11	2632	1	0.04	6016	0	0
12	2424	1	0.04	212	0	0
13	1860	0	0	2483	6	0.24
14	640	1	0.16	2032	1	0.04
15	2430	3	0.12	3021	0	0
16	1800	2	0.11	4952	16	0.34
17	960	0	0	3552	8	0.22
18	800	0	0	2300	0	0
19	4000	4	0.10	1861	4	0.22
20	200	2	1.0	3340	0	0
Inoculum	—	—	—	1861	0	0

(f) 15 litre batch fermenter studies

During batch growth of a 'normal' heterogeneous culture of Rm 5, the production of swarm cells occurs during the early and mid-exponential phase. On approaching stationary phase, swarm cell production is repressed, and most of the swarmers mature and develop to a certain extent before the onset of sporulation . Few single swarm cells are present in such a stationary phase culture. However, under certain conditions, and for no apparent reason, relatively high numbers of swarm cells are produced and accumulate in the stationary phase culture. Here it would appear that either repression of swarm cell production fails to occur, or their development is arrested. Behaviour of a culture in this manner has always been observed to prevent sporulation.

CO_2 has already been implicated as a possible 'trigger' for $P + S$ cell production and it would appear to play a major role in the regulation of other cellular expressions in Rhodomicrobium. In this section, experiments were performed to examine the behaviour of Rhodomicrobium under different batch conditions and in particular CO_2 tension. It was hoped to elucidate whether CO_2, light intensity and pH were stimuli for swarm cell production. Their effect on swarm cell development was also studied. The ability of Rhodomicrobium to sporulate is lost with continual batch subculturing, therefore attempts were made to induce sporulation by applying known environmental stimuli.

For the purpose of these experiments, a 15 litre batch fermenter was employed, the CO_2 tension of the culture being continuously monitored by the use of a submerged silicone coil connected directly to a katherometer. This apparatus proved ideal for monitoring cultural environments whilst investigating the cellular expression of Rhodomicrobium. Unfortunately time permitted only a few of the many possible experiments to be performed.

(i) Standard 'run'. ('Normal'cells, PYR/MAL medium, no pH control).

This was the 'control' run and involved growing Rm 5 under routine batch culture conditions. A log phase culture of 'normal' heterogeneous Rm 5 was used as inoculum. Routine growth medium (PYR/MAL + 0.1 M phosphate buffer, pH 6.8) was employed, and the cells incubated anaerobically at 30° C

with an incident light intensity of 9000 lux and low agitation (stirrer speed, 100 r.p.m.). Samples were removed at regular intervals and thoroughly examined. Figs. 3.26a, 3.26b, 3.26c, 3.26d and Plate 3.34 show the results obtained. Characteristic behaviour of the culture was apparent, and a 'normal' growth curve was obtained. The protein and bacteriochlorophyll contents of the culture increased at similar rates (Fig. 3.26b) and there was good correlation with dry weight (Fig. 3.26b.). Swarm cells were produced in large numbers during the exponential phase of growth and this was reflected by the increase in swarm cell total count (Fig. 3.26c), viable count (Fig. 3.26d) and dry weight values (Fig. 3.26b). The viable counts, however, were approximately a factor of 10 less than the total count values. It might be inferred that only 10% of the swarm cells are viable, but this contradicts observations of synchronized swarm cells developing in slide culture. The large difference between viable and total count values probably reflects the accuracy of the techniques involved. For a viable count, the swarm cells have to be separated manually by passage through a glass wool column, and whilst separation is excellent, a large proportion of swarm cells may be retained by the packing. Passage through the column, serial dilution and plating out all serve to reduce the viability of the cells. In contrast a total count of swarm cells in a heterogeneous culture can be estimated automatically and 'in situ' by the Coulter counter. An over-estimation in swarm cell number because of particle interference is feasible. A combination of these factors almost certainly contributes to the large discrepancy between the total and viable count values of the swarm cells in culture.

Variation in the mean swarm cell volume during the specific growth phases of the culture is apparent (Fig. 3.26c). The largest swarm cells (mean volume $0.90 \mu m^3$) were present during the mid-exponential growth phase. The 'Coulter profile' of such cells (Plate 3.34) consisted of a broad peak and a sizeable shoulder, the latter being indicative of rapid maturity and development. Development of the swarm cells appeared to be progressively inhibited after the mid-log growth phase until, by stationary phase, large numbers of small undeveloped swarm cells (Plate 3.34)

['Coulter profile' composed of a single tight peak (mean cell volume $0.72 \mu m^3$)] were present in culture.

The presence of P + S cells was not detected until late exponential phase ($\sim 80 h.$). By stationary phase, however, approximately 2% of the swarm cells gave rise to smooth colonies when plated out.

Both soluble nitrogen and oxygen tension remained constant for the duration of the run but there was a considerable increase in the concentration of dissolved CO_2 in the culture medium (Fig. 3.26a), presumably due to the ability of the cells to break down sodium pyruvate by the reactions of the TCA cycle. The soluble CO_2 tension maxima ($\sim 25\%$ v/v) occurred during late exponential phase ($\sim 80 h$) and apparently coincided with the appearance of P + S cells. The pH behaviour of the culture was typical, reaching a value of 8.1 by stationary phase, and a drop in the CO_2 tension of the culture was also apparent.
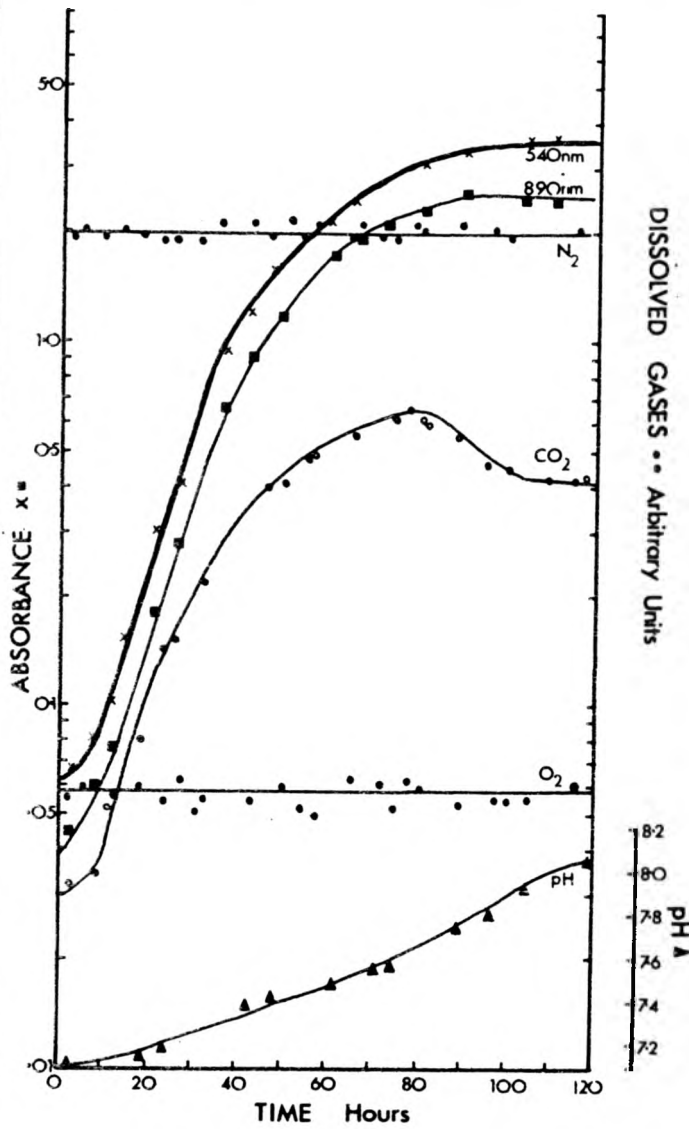
Fig. 3.26a

Properties exhibited by a batch culture of 'normal' heterogeneous Rm 5 cells grown in the 15 litre fermenter on PYR/MAL medium under routine conditions (30° C, 9000 lux, 100 r.p.m.).

(▲) Culture pH

(×) Culture absorbance (540 nm)

(■) Culture absorbance (890nm)

(o) Dissolved CO_2 tension

(•) Dissolved O_2 tension

(•) Dissolved N_2 tension

Fig. 3.26b

(o) Culture density. Absorbance 540 nm.

(•) Culture density. Dry weight (g/1).
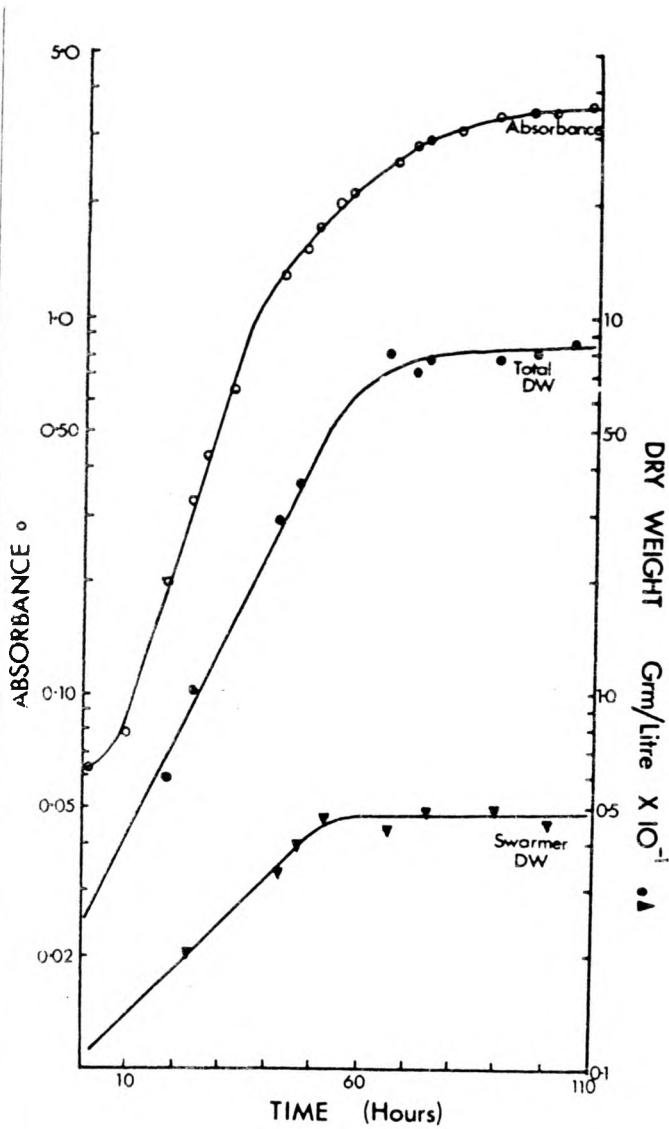
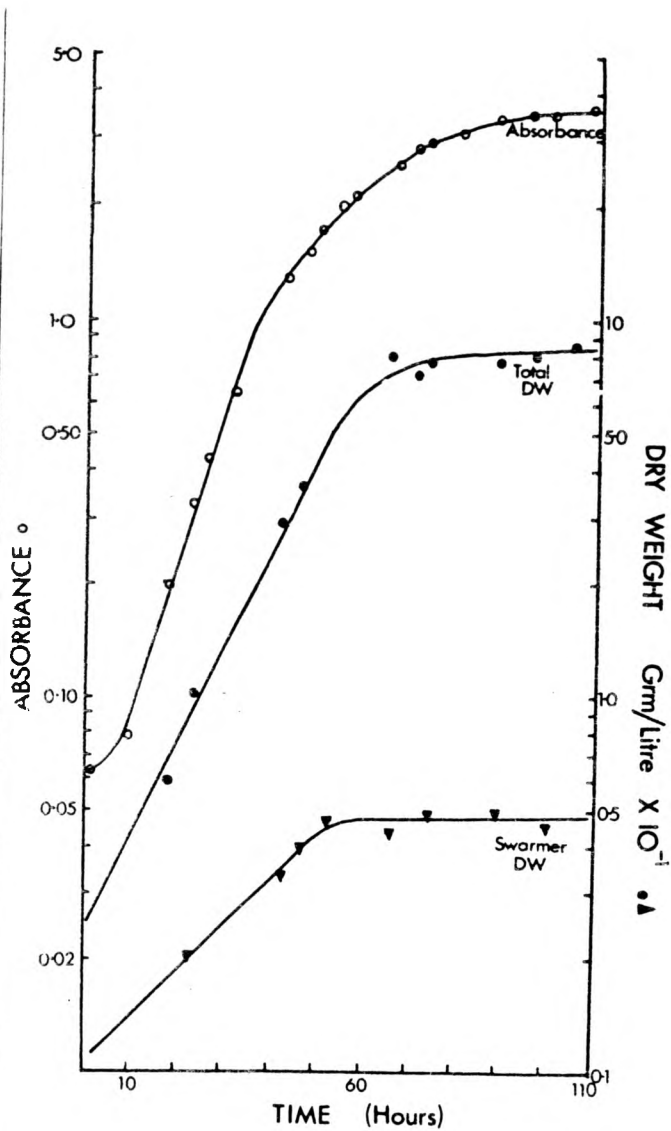(▼) Swarm cell density. Dry weight (g/1).

Fig. 3.26b

(o) Culture density. Absorbance 540 nm.

(●) Culture density. Dry weight (g/l).

(▼) Swarm cell density. Dry weight (g/l).

Fig. 3.26c

(X)   Culture density.   Absorbance 540 nm.

(▼)   Swarm cell count per ml. (Coulter counter).

(▽)   Mean swarm cell volume , μm^3.   (Coulter channelyser).

Fig. 3.26d

(X) Culture density. Absorbance 540 nm.

(●) Swarm cell viable count per ml.

(o) CO_2 tension in suspension. $\left(\dfrac{[CO_2]}{[CO_2]+[O_2]+[N_2]} \times 100 \right)$

Plate 3.3⁴

'Coulter profiles'

(A)   Mid ·exponential growth phase (48 h   ) swarm cells.

(B)   Swarm (single) cells in the culture stationary phase (140 h.).

(ii)   'Normal' heterogeneous Rm 5 cells. Batch culture at constant pH.

The behaviour of a 'normal' heterogeneous culture of Rm 5 growing at constant pH was followed. Routine growth medium (PYR/MAL + 0.1 M phosphate buffer, pH 6.8) was used, and the culture pH maintained at a value of 7.05 by the automatic addition of 0.1 M HCl. Cells were incubated anaerobically, stirrer speed 100 r.p.m., at 30° C with an incident light intensity of 9000 lux.

The fact that the cells were grown at constant pH had little effect on the shape of the culture growth curve (Fig. 3.27a). pH limitation was unlikely to be responsible for the gradual slowing down of cell growth rate at high culture density and light limitation is implicated. A close correlation existed between the soluble CO_2 tension and culture growth (Fig.3.27a) and a high soluble CO_2 tension (57%) (v/v) accumulated by stationary phase. There was no reduction in the soluble CO_2 tension during late growth. A drop in the number of swarm cells present was apparent (Fig.3.27b) in comparison to the 'control' experiment (previous 'run'). It would appear, therefore, that swarm cell production is affected by growth at low and/or constant pH.   The 'Coulter profile' of stationary phase swarm cells was similar to that of log phase swarm cells (Plate 3.35) and a broad peak and sizeable shoulder can be observed in both cases. Unlike swarm cells from the stationary phase of the 'control' culture, development was not inhibited. The conditions of pH control were conducive to the production and development of swarm cells throughout growth. In the absence of pH control swarm cell production occurs, but their development is arrested. Although development of swarm cells was apparent throughout the growth of the culture at constant pH, considerable differences in the swarm cell volume were still evident during the culture growth phases (Plate 3.35). The mean volume of the swarm cells present in suspension increased during the early exponential phase of the culture to a maximum of $1.04 \, \mu m^3$, and then decreased rapidly to $0.72 \, \mu m^3$ by stationary phase. The volume of the swarm cells produced appeared to be governed by the growth rate of the mother cells.
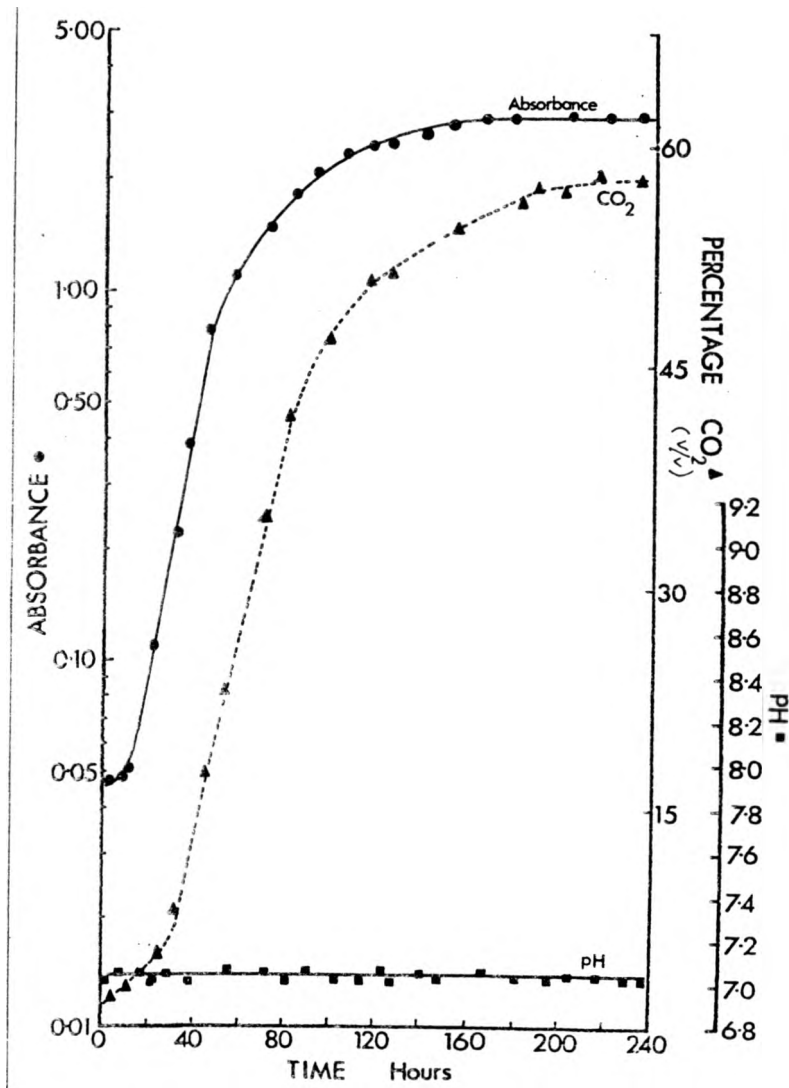
Fig. 3.27a

Properties exhibited by a batch culture of 'normal' Rm 5 cells grown
in the 15 litre fermenter on PYR/MAL medium at constant pH (7.05)
(30° C, 9000 lux, 100 r.p.m.).

(●) Culture density. Absorbance 540 nm.

(▲) CO_2 tension in suspension.
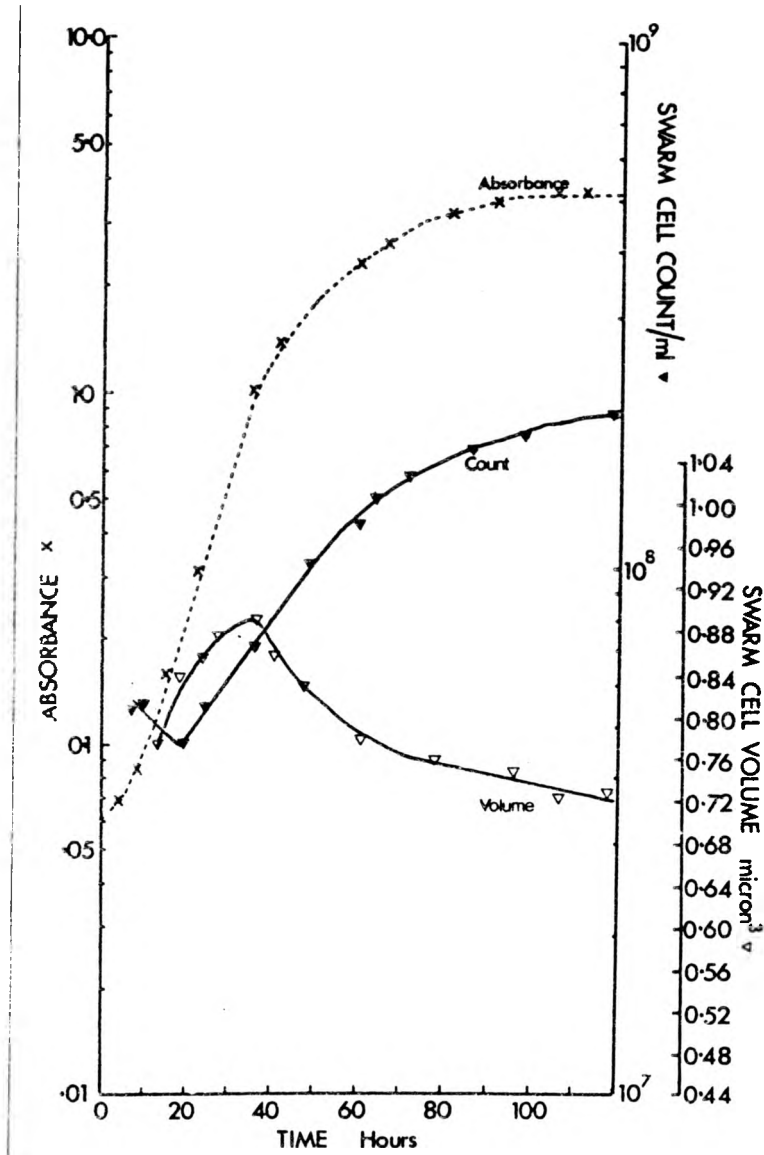
(■) pH.

Fig. 3.27b

(●)   Culture density.   Absorbance 540 nm.

(o)   Swarm cell count per ml.   (Coulter counter).

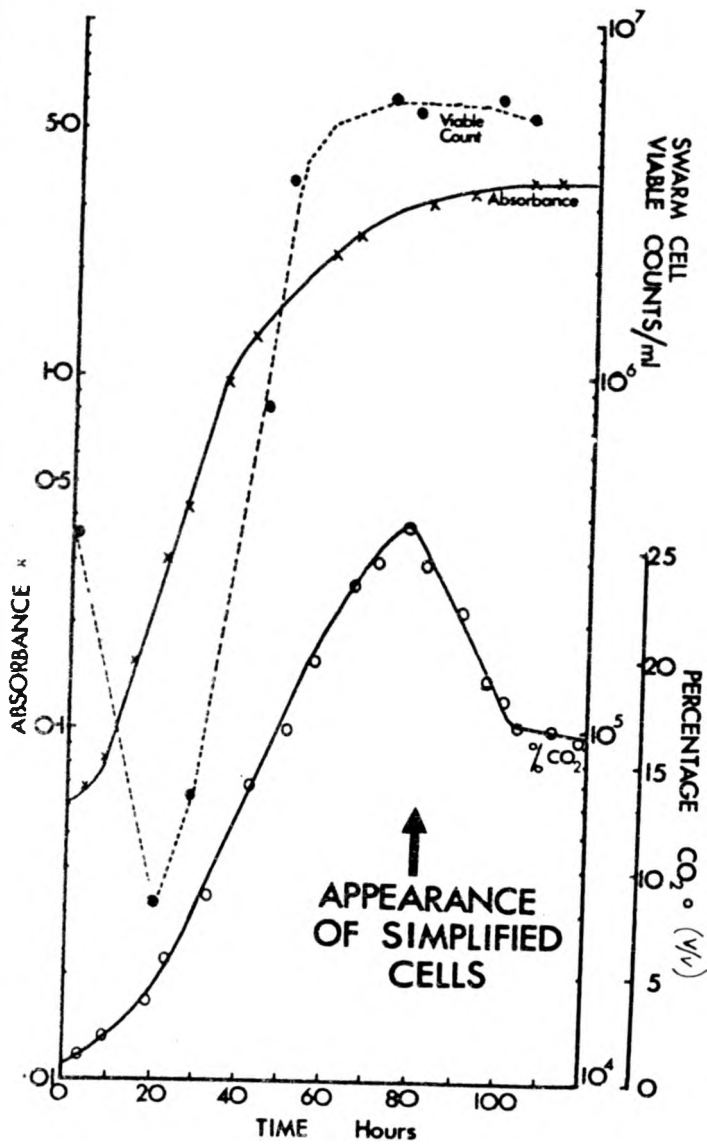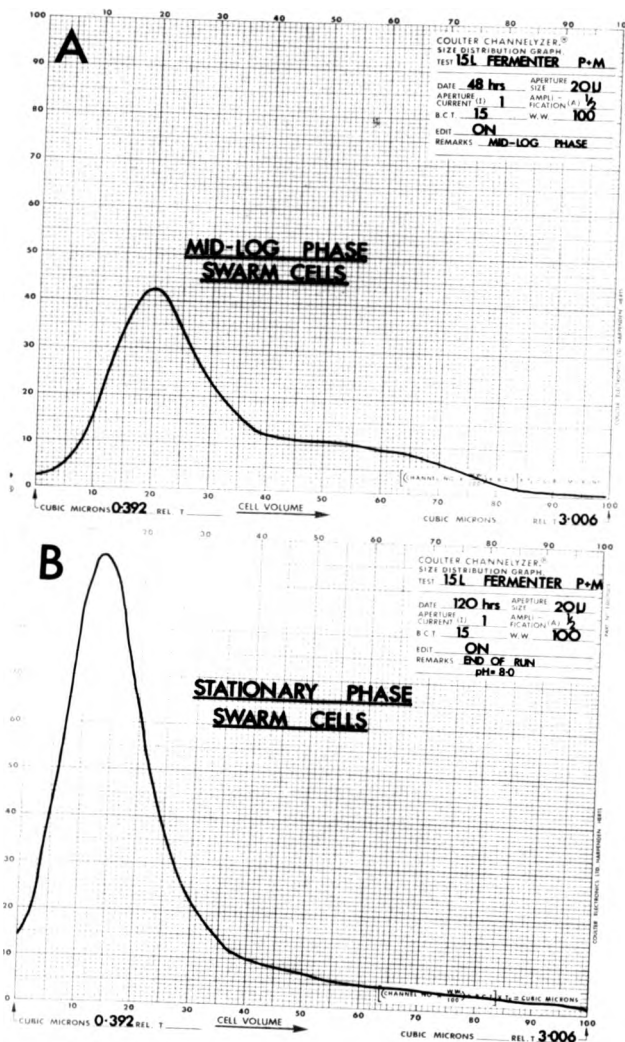(Δ)   Mean swarm cell volume, μm^3.   (Coulter channelyser).

Plate 3.35

'Coulter profiles'

(A) Mid-exponential growth phase (60 h   ) swarm cells.

(B) Swarm (single) cells in the stationary phase culture (220 h   ).

(iii) 'Normal' heterogeneous Rm 5 cells. Batch culture on sodium
hydrogen malate (MAL) medium.

The growth of <u>Rm 5</u>, under conditions of relatively low CO_2 tension, on
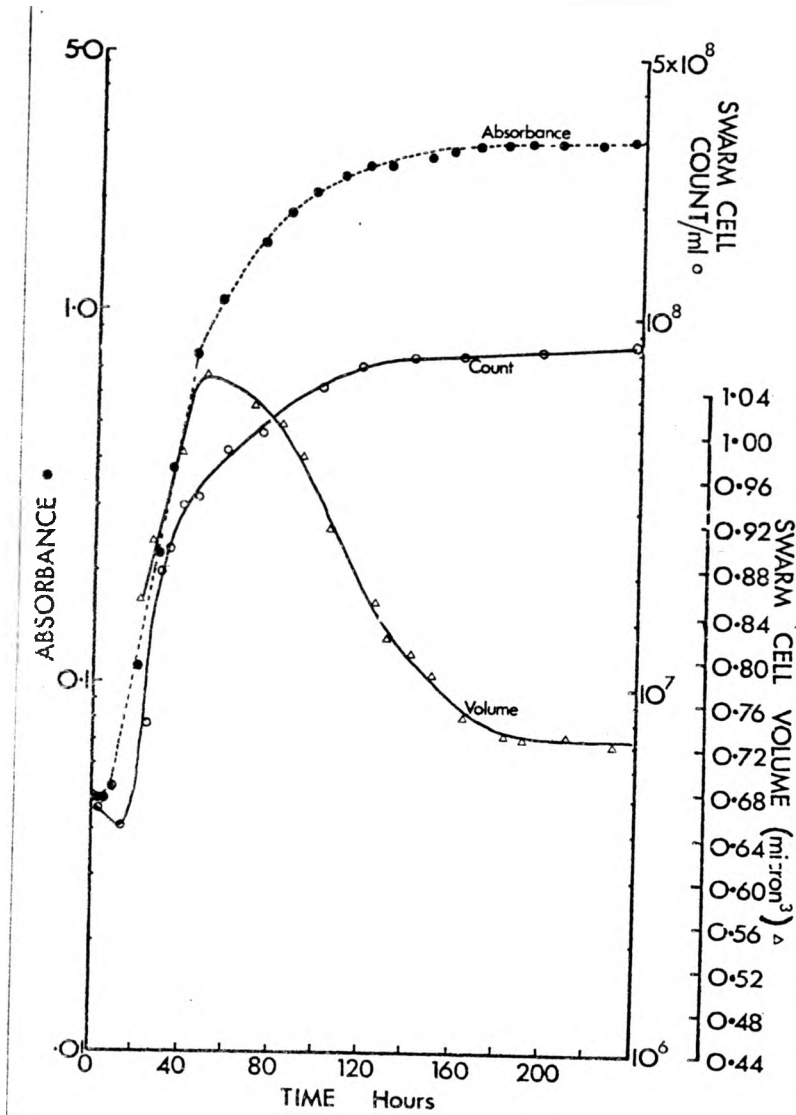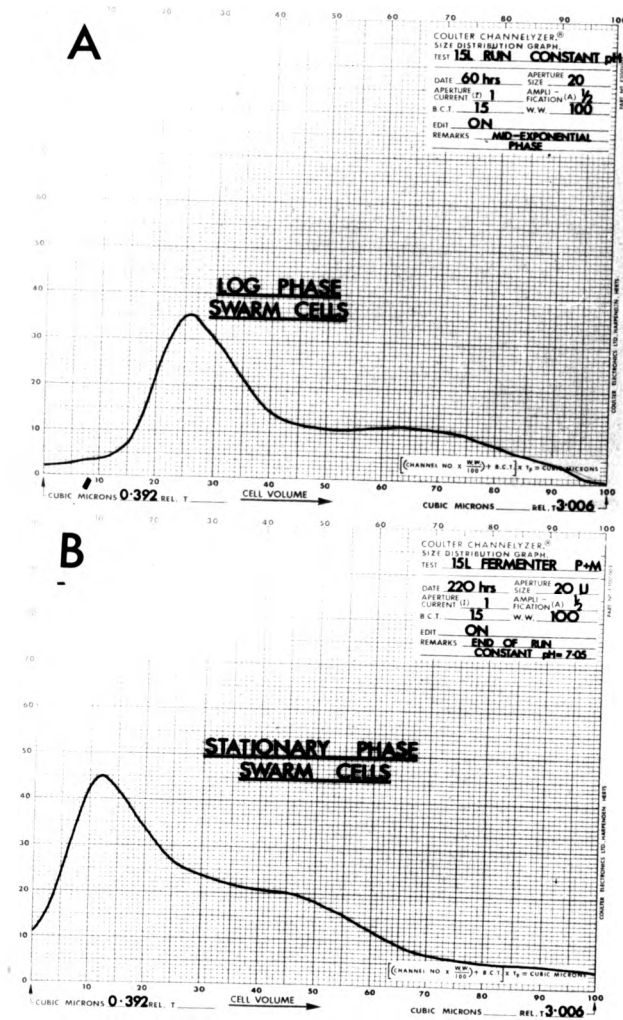medium employing sodium hydrogen malate as sole carbon source is
known to enhance sporulation (C.S. Dow, this laboratory, personal
communication).   In contrast, sporulation of <u>B. subtilis</u> is repressed
by growth on medium containing L. malate (Ohné and Rutberg, 1976).

In this experiment, the behaviour of a 'normal' heterogeneous culture
of <u>Rm 5</u> growing on limiting malate growth medium was followed. Normal
salts medium containing sodium hydrogen malate (1.5 g/l) as sole carbon
source and 0.1 M phosphate buffer (pH 6.8) was employed.   The culture
was incubated anaerobically as usual (30° C, 9000 lux, 100 r.p.m.).

Little difference was observed in the morphology of the cells grown
under these conditions, and exospores were not produced. A normal
growth curve with a low stationary phase culture density was obtained,
reflecting the reduction in the carbon concentration of the medium (Fig. 3.28a).
The soluble CO_2 tension of the culture was relatively low throughout. A
maximum value of 10% CO_2 (v/v) had accumulated by late exponential phase, but
by stationary phase only 7% (v/v) was detectable, due to the pH effect on the
CO_2/bicarbonate equilibrium.   Swarm cells were produced prolifically
throughout culture growth, motility being retained right through to
stationary phase. The alteration of carbon source and reduction   in the
carbon dioxide concentration had little effect on the number of single cells
present in the stationary phase (Fig. 3.28b). 2.18×10^8/ml were present
here as opposed to 2.0×10^8/ml in the control experiment where PYR/MAL
medium was employed. This similarity may be attributed to the greater
and faster increase in culture pH under limiting malate conditions, whereby
production of swarm cells is unaffected, but inhibition of development is
advanced, resulting in a greater number of swarm cells accumulating.
Swarm cells from the mid-exponential phase of this culture (culture
absorbance 540 nm, 1.22, pH 7.7) had a 'Coulter profile' (Plate 3.36)
which suggested inhibition of development. However, swarm cells from the
standard run at a similar growth phase (culture absorbance 1.26, pH 7.4)

(iii) 'Normal' heterogeneous Rm 5 cells. Batch culture on sodium
hydrogen malate (MAL) medium.

The growth of Rm 5, under conditions of relatively low CO_2 tension, on
medium employing sodium hydrogen malate as sole carbon source is
known to enhance sporulation (C.S. Dow, this laboratory, personal
communication). In contrast, sporulation of B. subtilis is repressed
by growth on medium containing L. malate (Ohné and Rutberg, 1976).

In this experiment, the behaviour of a 'normal' heterogeneous culture
of Rm 5 growing on limiting malate growth medium was followed. Normal
salts medium containing sodium hydrogen malate (1.5 g/l) as sole carbon
source and 0.1 M phosphate buffer (pH 6.8) was employed. The culture
was incubated anaerobically as usual (30° C, 9000 lux, 100 r.p.m.).

Little difference was observed in the morphology of the cells grown
under these conditions, and exospores were not produced. A normal
growth curve with a low stationary phase culture density was obtained,
reflecting the reduction in the carbon concentration of the medium (Fig. 3.28a).
The soluble CO_2 tension of the culture was relatively low throughout. A
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stationary phase. The alteration of carbon source and reduction in the
carbon dioxide concentration had little effect on the number of single cells
present in the stationary phase (Fig. 3.28b). 2.18 x 10^8/ml were present
here as opposed to 2.0 x 10^8/ml in the control experiment where PYR/MAL
medium was employed. This similarity may be attributed to the greater
and faster increase in culture pH under limiting malate conditions, whereby
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advanced, resulting in a greater number of swarm cells accumulating.
Swarm cells from the mid-exponential phase of this culture (culture
absorbance 540 nm, 1.22, pH 7.7) had a 'Coulter profile' (Plate 3.36)
which suggested inhibition of development. However, swarm cells from the
standard run at a similar growth phase (culture absorbance 1.26, pH 7.4)

were the same size, but still showed signs of significant development. The volume changes of the swarm cells followed the usual pattern during culture growth (Fig. 3.28b). The largest swarm cells (mean volume 0.93 μm^3) were present during the log phase of growth, and their size decreased steadily with culture age (stationary phase mean volume = 0.62 μm^3).

The numbers of P + S cells appearing were reduced by 'normal' culture growth on limiting malate medium. Less than 0.1% of the swarm cells present in the late exponential phase culture gave rise to smooth colonies when plated out.
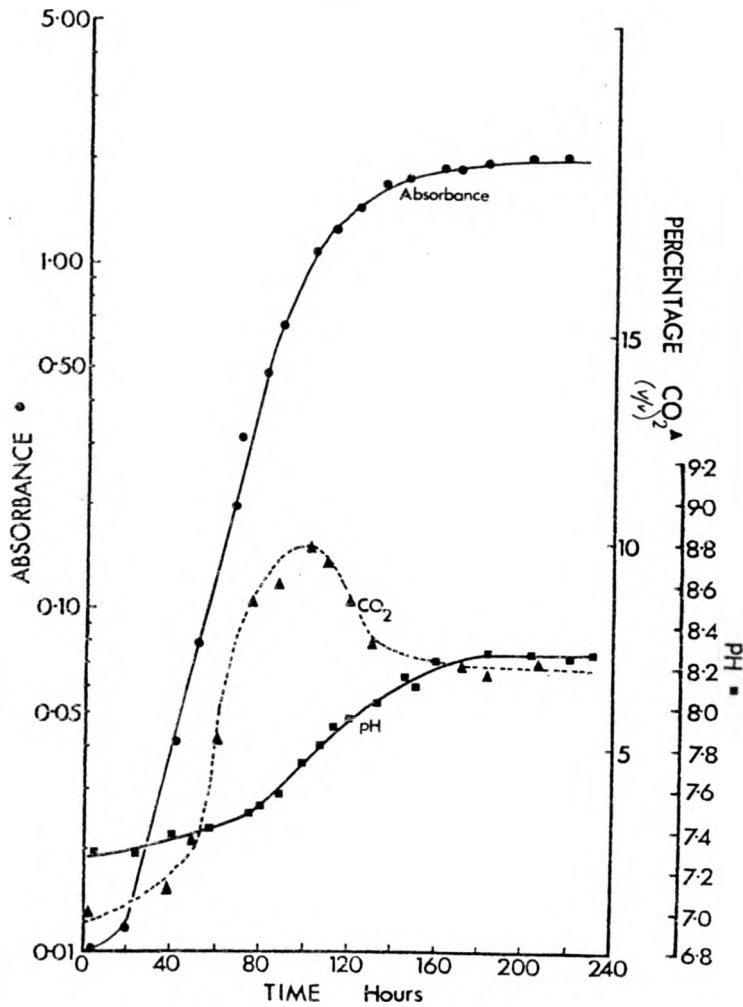
Fig. 3.28a

Properties exhibited by a batch culture of 'normal' heterogeneous Rm 5
grown in the 15 litre fermenter on MAL medium under routine conditions
$(30^{o} C, 9000$ lux, 100 r.p.m.).

(●)   Culture density. Absorbance 540 nm.

(▲) CO_2 tension in suspension.
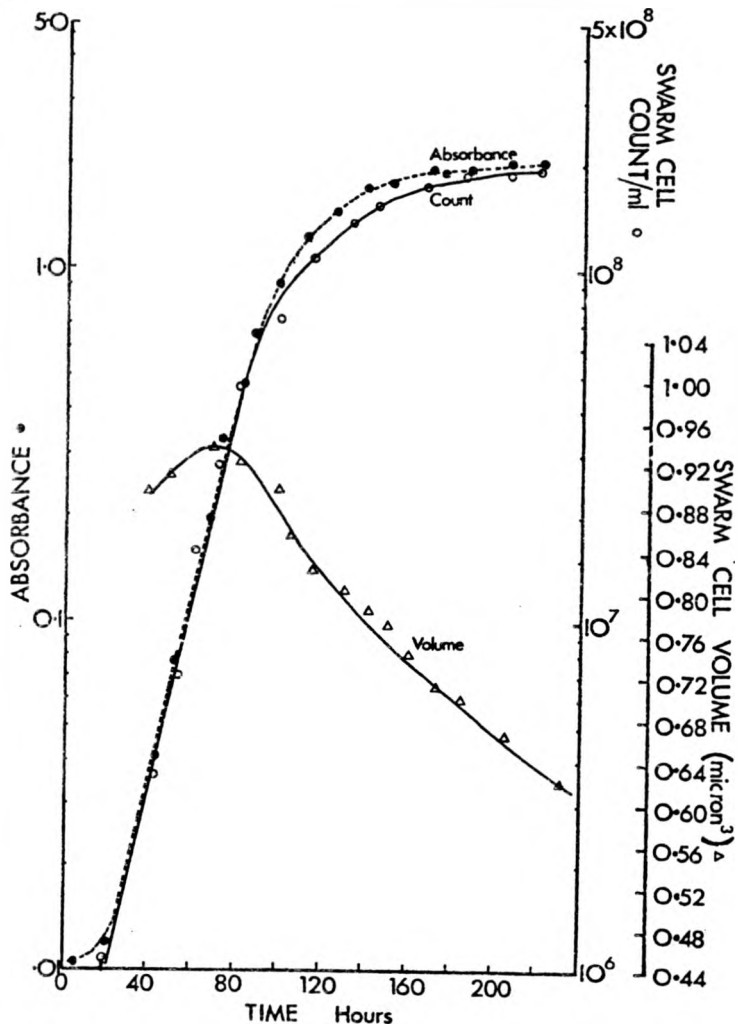
(■)   Culture pH.

Fig. 3.28b

(●)   Culture density.   Absorbance 540 nm.

(o)   Swarm cell count per ml.   (Coulter counter).

(△)   Mean swarm cell volume, μm^3.   (Coulter channelyser).

Plate 3.36

The 'Coulter profile' (solid line) of swarm cells from the exponential
growth phase (100 h   ) of the culture grown on MAL medium compared
with that (dotted line) of the corresponding swarm cells from a culture
of similar density grown on PYR/MAL medium.

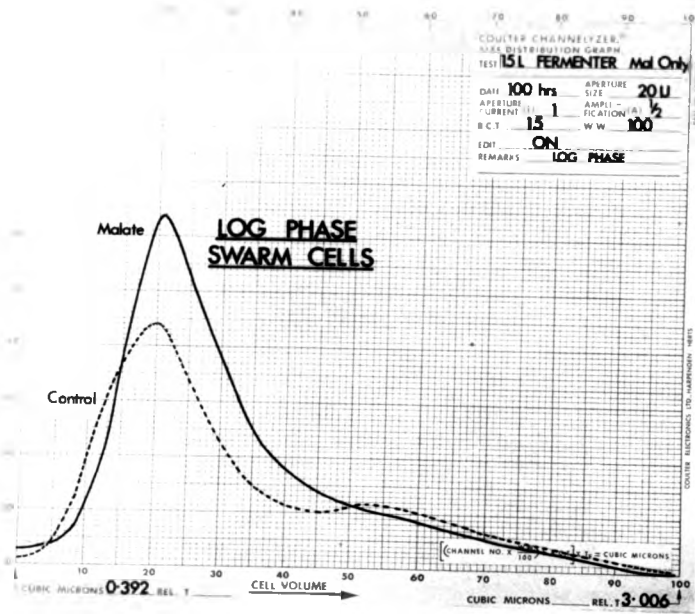(iv) 'Normal' heterogeneous Rm 5 cells. Batch culture on sodium
hydrogen malate (MAL) medium. CO_2 tension reduced.

The effect of growing 'normal' heterogeneous Rm 5 under low CO_2
tension was studied.

The culture was allowed to establish itself on normal salts medium
containing sodium hydrogen malate (1.5g/l) and 0.1 $\underline{M}$ phosphate buffer
(pH 6.8) under the usual anaerobic growth conditions (30° C, 9000 lux,
100 r.p.m.). The culture abosrbance 540 nm was allowed to reach 0.15
before oxygen-free nitrogen was continuously sparged through the culture
at a flow rate of 40 ml/min. A gradual reduction in the soluble CO_2
tension occurred (Fig. 3.29a). Removal of the carbon dioxide and the
buffering effect of bicarbonate enabled the pH to rise to a high value.
This, however, had no effect on the stationary phase culture density.
'Normal' cell morphology was observed throughout and sporulation was
not induced. A reduction of at least 50% in the swarm cell count was,
however, evident (Fig. 3.29b). The 'Coulter profile' (Plate 3.37) of
swarm cells from the mid-log phase culture (absorbance 540 nm, 0.74,
pH 7.7) indicated little development. This observation, together with
the swarm cell volume and count graphs (Fig.3.29b) suggests that
inhibition of swarm cell production is occurring during the mid-exponential
phase of culture growth. It can therefore be concluded that conditions of
high pH and/or low CO_2 tension interfere with both swarm cell production
and subsequent development.

(iv) 'Normal' heterogeneous Rm 5 cells. Batch culture on sodium
     hydrogen malate (MA L) medium. CO_2 tension reduced.

The effect of growing 'normal' heterogeneous Rm 5 under low CO_2
tension was studied.

The culture was allowed to establish itself on normal salts medium
containing sodium hydrogen malate (1.5 g/l) and 0.1 M phosphate buffer
(pH 6.8) under the usual anaerobic growth conditions (30° C, 9000 lux,
100 r.p.m.). The culture abosrbance 540 nm was allowed to reach 0.15
before oxygen-free nitrogen was continuously sparged through the culture
at a flow rate of 40 ml/min. A gradual reduction in the soluble CO_2
tension occurred (Fig. 3.29a). Removal of the carbon dioxide and the
buffering effect of bicarbonate enabled the pH to rise to a high value.
This, however, had no effect on the stationary phase culture density.
'Normal' cell morphology was observed throughout and sporulation was
not induced. A reduction of at least 50% in the swarm cell count was,
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swarm cells from the mid-log phase culture (absorbance 540 nm, 0.74,
pH 7.7) indicated little development. This observation, together with
the swarm cell volume and count graphs (Fig. 3.29b) suggests that
inhibition of swarm cell production is occurring during the mid-exponential
phase of culture growth. It can therefore be concluded that conditions of
high pH and/or low CO_2 tension interfere with both swarm cell production
and subsequent development.

Fig. 3.29a

Properties exhibited by a batch culture of 'normal' heterogeneous Rm 5
cells grown in the 15 litre fermenter on MAL medium under conditions
of reduced CO_2 tension. (N_2 sweep 40 ml/min). (30° C, 9000 lux,
100 r.p.m.).

(●) Culture density. Absorbance 540 nm.

(▲) CO_2 tension in suspension.
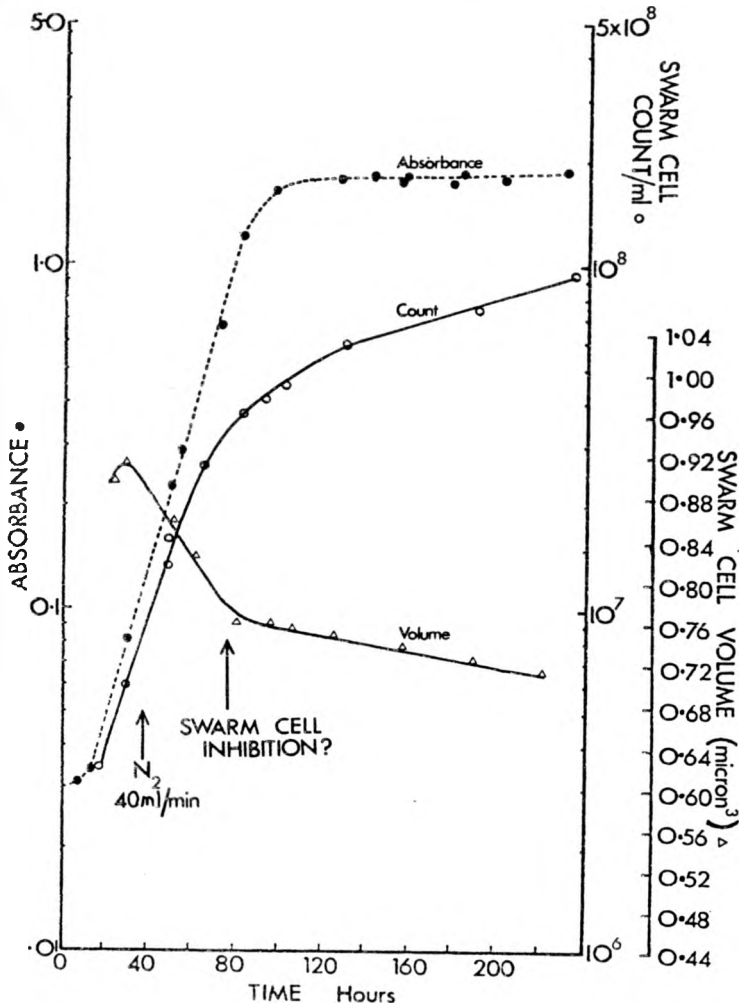
(■) Culture pH.

Fig. 3.29b

(●) Culture density. Absorbance 540 nm.

(○) Swarm cell count per ml. (Coulter counter).

(△) Mean swarm cell volume, μm^3. (Coulter channelyser).
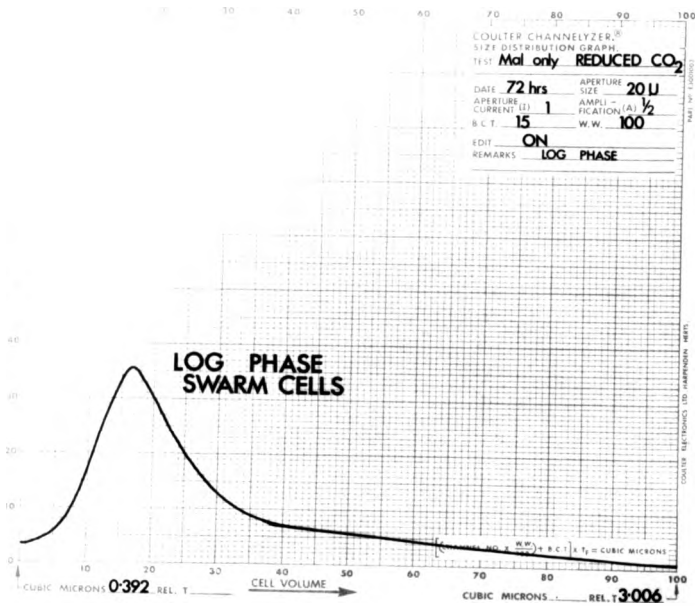
Plate 3.37

The 'Coulter profile' of swarm cells from the exponential growth phase (72 h).

(v) 'Normal' heterogeneous Rm 5 cells. Batch culture on sodium hydrogen
 <u>malate (MAL) growth medium. CO_2 tension greatly reduced.</u>

The growth conditions employed here were identical to those of the
previous experiment, except that oxygen-free nitrogen was sparged
through the culture at the higher rate of 90 ml/min. The reduction in the
soluble CO_2 tension was more rapid and substantial, and a greater
stationary phase pH was evident (Fig. 3.30a). As a consequence, the
stationary phase culture density was slightly reduced due to pH limitation
of growth. High pH, however, and very low CO_2 tension, did not induce
sporulation. A slight drop in the number of swarm cells produced by
the culture was apparent (Fig.3.30b) and it might be inferred that the
further reduction in the CO_2 tension is responsible. However, it is more
likely to be an insignificant observation associated with pH limitation of
culture growth. The slight reduction in the swarm cell count towards
the end of the experiment (Fig.3.30b) is almost certainly due to lysis and
not their development, which ceases during the early culture growth. The
'Coulter profile' of swarm cells present during the mid-exponential phase
of the culture growth once again indicates little sign of development
(Plate 3.38). The mean volumes of the swarm cells present during the
specific growth phases of the culture were similar to values obtained in
the previous experiment. i.e. the volume of the swarm cells present was
maximal (0.96 μm^3) during early culture growth, and then progressively
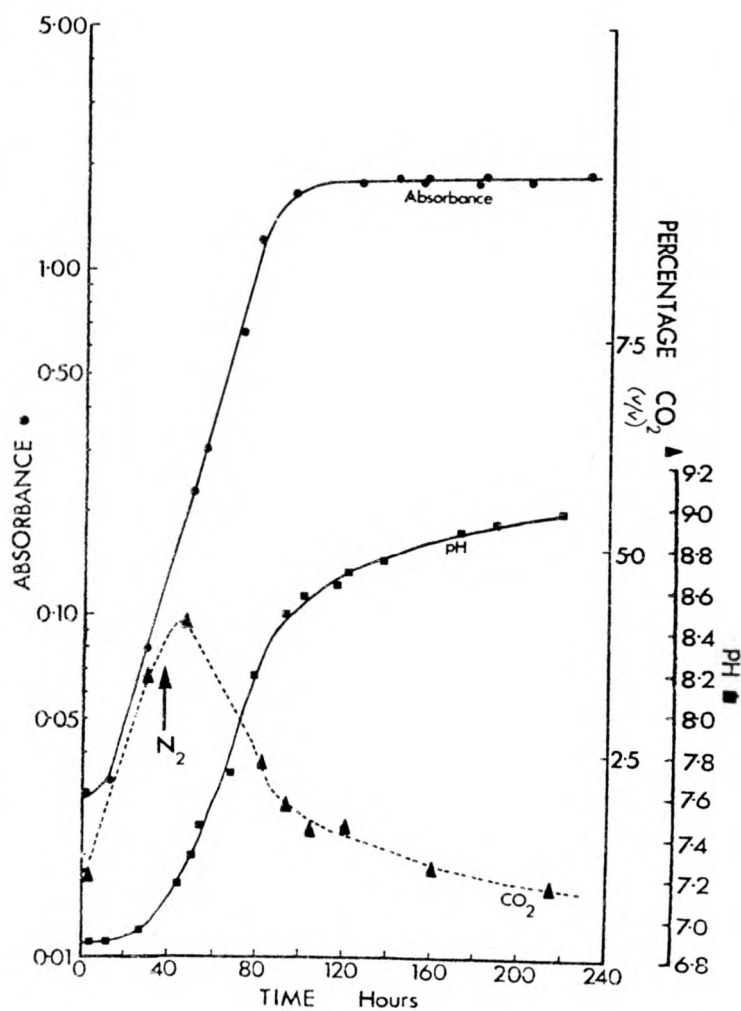decreased to a minimum value of 0.69 μm^3 by stationary phase.

Fig. 3.30a

Properties exhibited by a batch culture of 'normal' heterogeneous Rm 5
cells grown in the 15 litre batch fermenter on MAL medium under
conditions of greatly reduced CO_2 tension. (N_2 sweep 90 ml/min).
(30° C, 9000 lux, 100 r.p.m.).

(●) Culture density. Absorbance 540 nm.

(▲) CO_2 tension in suspension.
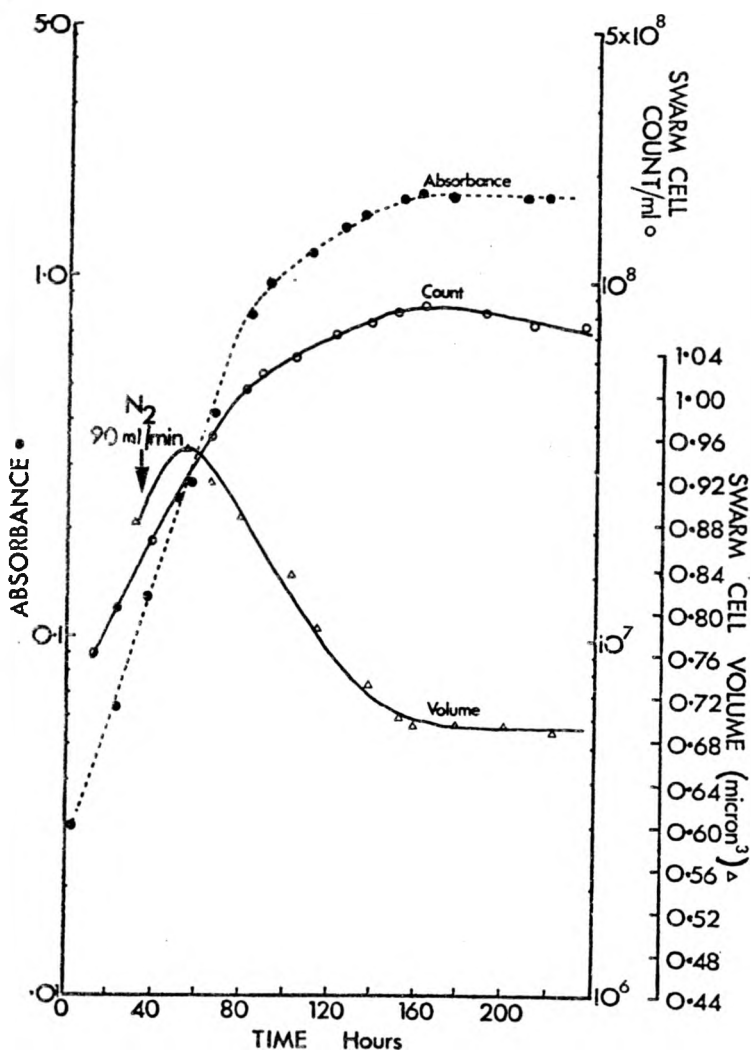
(■) Culture pH.

Fig. 3.30b

(●) Culture density. Absorbance 540 nm.

(○) Swarm cell count per ml. (Coulter counter).

(△) Mean swarm cell volume, μm^3. (Coulter channelyser).
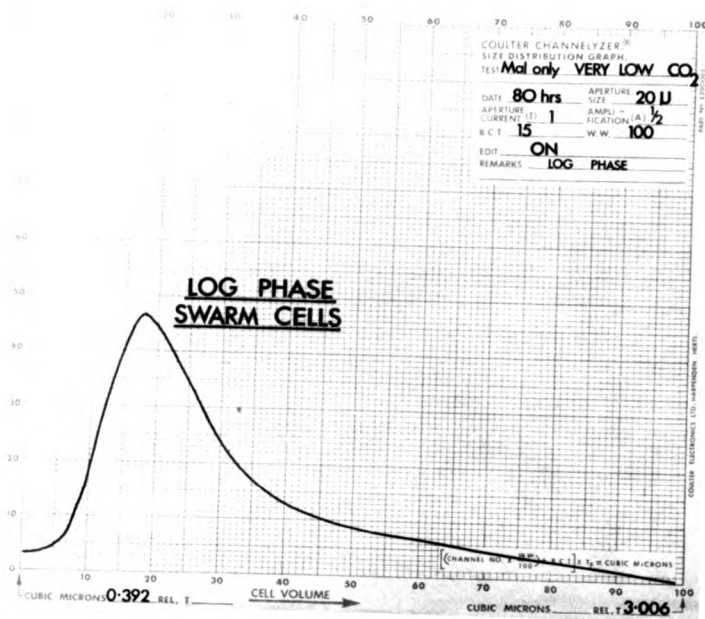
Plate 3.38

The 'Coulter profile' of swarm cells from the exponential growth
phase (80 h.).

(vi) 'Normal' heterogeneous Rm 5 cells. Batch culture on sodium
     hydrogen malate (MAL) medium in the presence of trace elements.

Since it has been suggested that the presence of trace elements is a
necessary requisite for successful sporulation in Bacilli (Hanson, personal
communication; Freese, 1972), it was decided to investigate the effect
of growing Rm 5 on malate medium (MAL) supplemented with Pfennig's
trace element solution (Pfennig, 1969). The culture was incubated
anaerobically under the usual conditions (30° C, 9000 lux, 100 r.p.m.).
Fig.3.31a shows the results obtained. The behaviour of the culture, with
respect to pH, CO_2 and density was unaffected by trace elements and
comparable to the earlier 'run' (iii) performed in the absence of trace
elements. In contrast, however, sporulation did occur, the first exo-
spores being observed during the late exponential phase of growth
(140 h) with large numbers of motile immature swarm cells still present.
Earlier studies (Dow, 1974) suggested that sporulation of Rm 5 only
occurred when swarm cell production had been terminated, and the
development of most of the swarm cells had been completed. In this
experiment, however, swarm cell development and production had been
inhibited before onset of sporulation, resulting in both motile swarm
cells and exospores being present together. A very high 'swarm cell'
count was apparent (Fig.3.31b), but this is not a true reflection of the
situation. Although the morphology of exospores and swarm cells (from a
stationary phase culture) are vastly different, they have very similar
volumes and cannot be distinguished from one another by the 'Coulter'
counter and channelyser (Plate 3.39). Any count therefore represents
the sum of both the swarm cell and exospore numbers. The graph of
swarm cell volume against culture age is typical (Fig.3.31b), with the
largest swarm cells being present during the mid-exponential phase of
culture growth. However, a rapid levelling out of the curve occurs at
the same time as the onset of sporulation, at which time the graph ceases
to be singularly representative.

5·00
1·00
0·50
0·10
0·05
0·01
ABSORBANCE ●
0
40
80
120
160
200
240
TIME Hours
Absorbance
CO_2
pH
15
10
5
PERCENTAGE CO_2 (v/v) ▲
9·2
9·0
8·8
8·6
8·4
8·2
8·0
7·8
7·6
7·4
7·2
7·0
6·8
pH ■
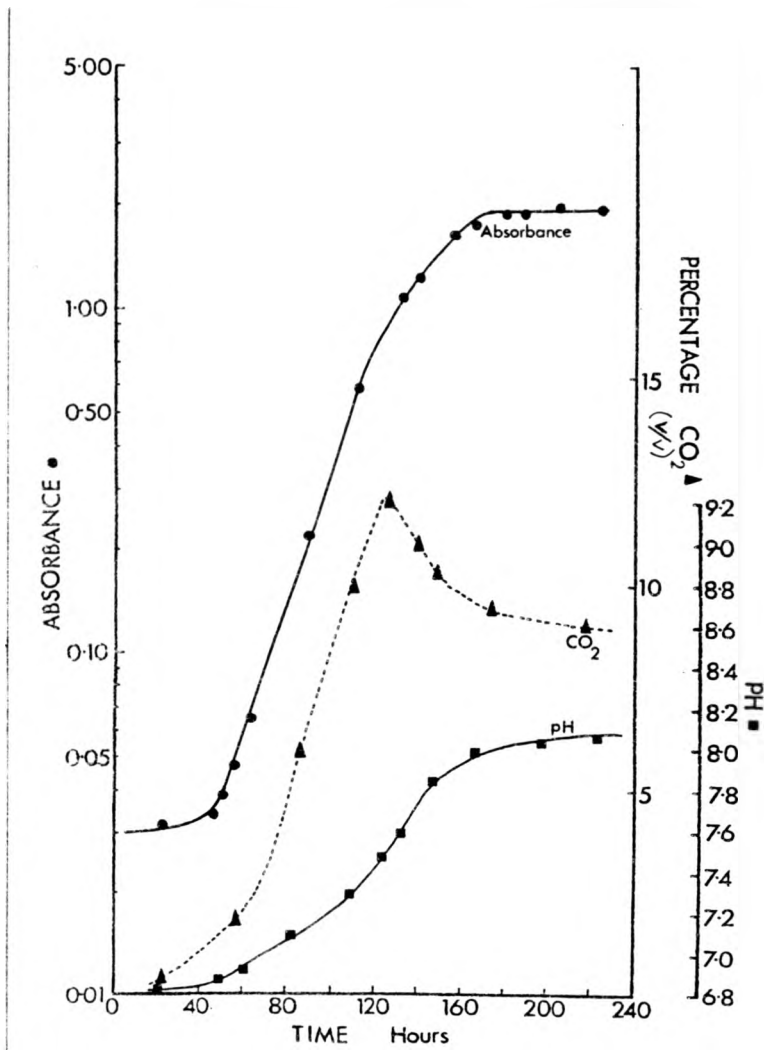
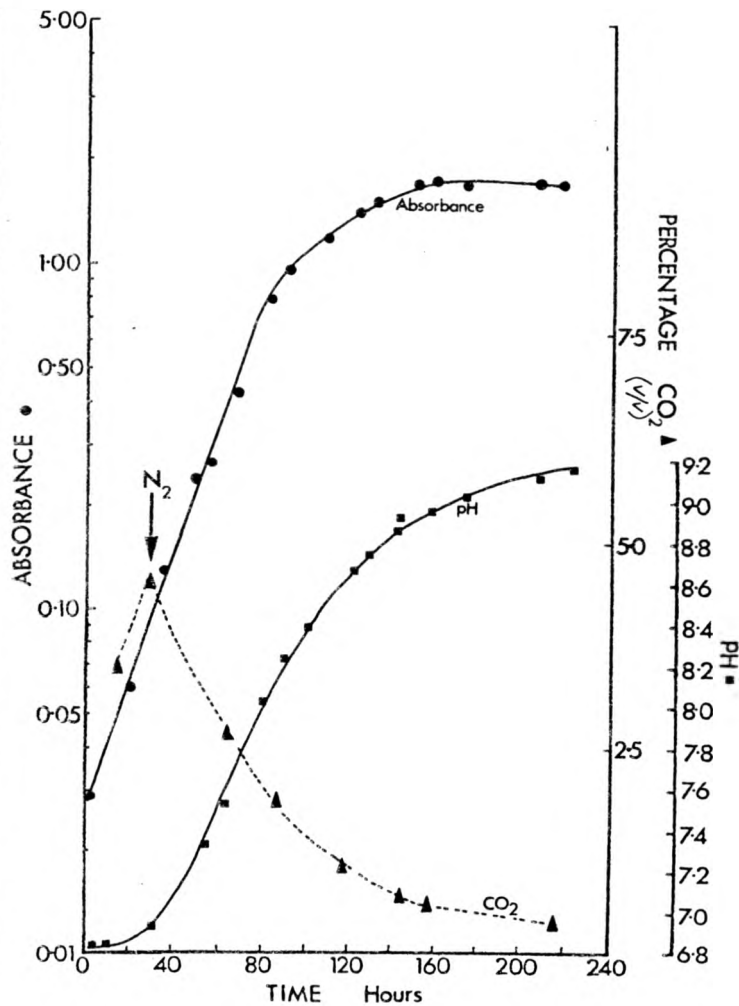

Fig. 3.31a

Properties exhibited by a batch culture of 'normal' heterogeneous Rm 5 cells grown in the 15 litre fermenter on MAL medium, supplemented with trace elements. (30° C, 9000 lux, 100 r.p.m.).

(●) Culture density. Absorbance 540 nm.

(▲) CO_2 tension in suspension.
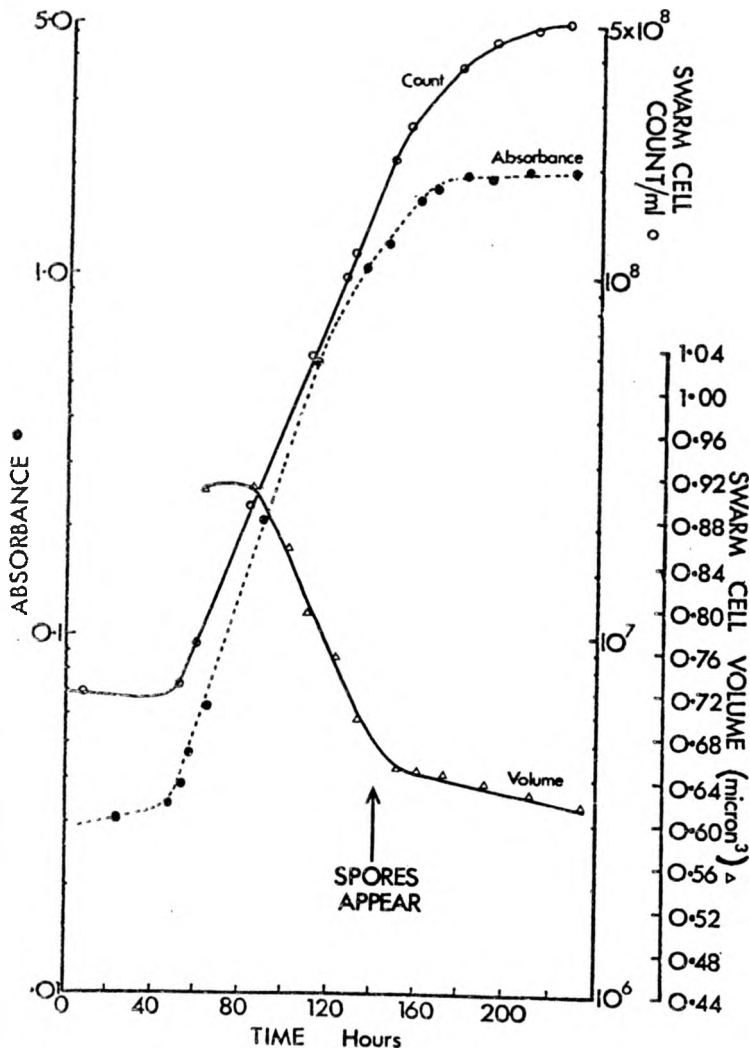
(■) Culture pH.

Fig. 3.31b

(●) Culture density. Absorbance 540 nm.

(o) Swarm cell count (+spore count) per ml. (Coulter counter).

(△) Mean swarm cell volume (spore volume) μm^3. (Coulter channelyser).

The chart shows a Coulter Channelyzer size distribution graph with two curves labelled "EXOSPORE PREPARATION" (solid line) and "SWARM CELLS (STAT PHASE)" (dotted line). The form header reads: COULTER CHANNELYZER. SIZE DISTRIBUTION GRAPH. TEST PURIFIED SPORE PREP. DATE JAN 1976. APERTURE SIZE 20 µ. APERTURE CURRENT (I) 1. AMPLIFICATION (A) ½. B.C.T. 15. w.w. 100. EDIT ON. REMARKS. Axis values 0·392 and 3·006.

Plate 3.39

The 'Coulter profile' (dotted line) of the single cells (swarm cells + exospores) present in the stationary phase culture, compared with that of a purified exospore preparation (solid line).

Summary of the behaviour of 'normal' heterogeneous Rm 5 in the 15 litre
batch fermenter experiments

The major features concerning the behaviour of 'normal' heterogeneous
Rm 5 cells in the 15 litre batch fermenter under different environmental
conditions are displayed in Table 3.10. By variation of the culture
medium and the incubation conditions, it was possible to alter the
dissolved carbon dioxide tension considerably. The growth rate of the
cells was affected by the medium employed and also by the pH (possibly
reflecting the CO_2 tension). Fastest culture growth occurred on PYR/MAL
medium at low constant pH, whereas slowest growth was evident on MAL
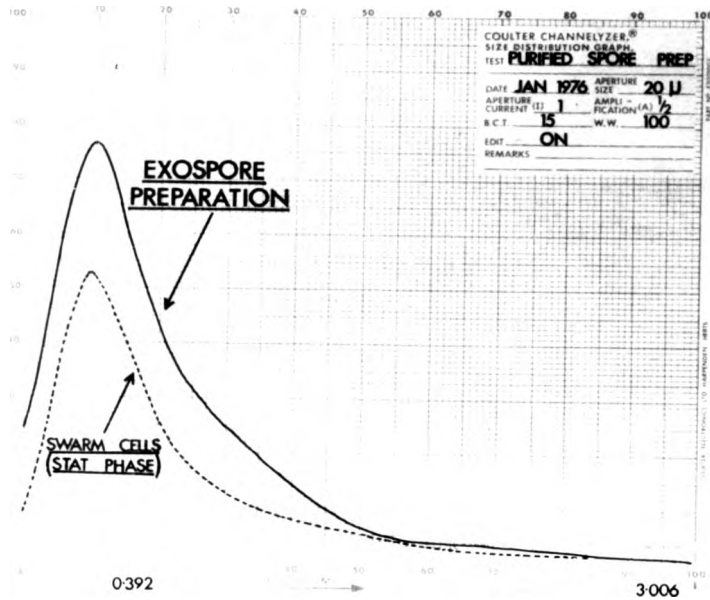medium at high pH (N_2 sweep 90 ml/min). The importance of CO_2 as a
possible control factor in cellular expression is apparent since the swarm
cells giving rise to smooth colonies (P + S cells) occur at times of highest
CO_2 tension. The frequency of occurrence of such cells was reduced
when Rm 5 was cultured at lower CO_2 tensions, i.e. on MAL medium.
Results indicate that the numbers of swarm cells produced might also be
proportional to the amount of CO_2 in solution. However, the fact that
swarm cell development is inhibited to varying extents by environmental
conditions makes this difficult to ascertain, i.e. the number of swarm
cells present in culture is not determined solely by their rate of production
but also their rate of development. A culture that produces a large
number of rapidly developing swarm cells and a culture that produces a
low number of slowly developing swarm cells would be expected to give
similar swarm cell counts. Maturation and development of immature
swarm cells appear to be adversely affected by high pH. This observation,
together with previous data, implicates both high pH and low light intensity
as repressors of swarm cell development. Different culture conditions
seem to have little effect on the size of the swarm cells produced. The
largest swarm cells (mean volume 0.90-1.1 μm^3) are always produced
during the early to mid-exponential phase of culture growth. By stationary
phase, however, the size of the single cells present in suspension has
dropped considerably (mean volume 0.60-0.74 μm^3).

Exospore formation occurs when the cells are grown in the presence of
trace elements. Continual subculturing on medium lacking trace elements
results in their gradual loss of ability to sporulate.

	RUN (i) PYR/MAL	RUN (ii) PYR/MAL Const. pH	RUN (iii) MAL	RUN (iv) MAL N_2 40 ml/min	RUN (v) MAL N_2 90 ml/min	RUN (vi) MAL +T.E.
MEAN DOUBLING TIME (h)	7.0	5.0	11.8	13.0	16.0	13.0
STATIONARY PHASE ABSORBANCE	3.5	3.2	2.1	2.06	1.6	2.06
STATIONARY PHASE pH	7.95	7.1	8.2	8.95	9.5	8.1
PERCENTAGE CO_2 MAX (v/v)	24.4	57.0	10.0	4.6	4.4	13.0
PERCENTAGE CO_2 FINAL (v/v)	15.6	57.0	7.0	0.8	0.0	9.0
SWARM CELL COUNT/ml	2.0×10^8	8.8×10^7	2.2×10^8	1.1×10^8	7.5×10^7	6.2×10^8
PERCENTAGE P+S	2.0	–	0.1	–	0.0	–
SWARM CELL Max vol (μm^3)	0.90	1.11	0.92	0.92	0.95	0.92
SWARM CELL Final vol +	0.72	0.74	0.60	0.71	0.71	0.61
DEVELOPMENT	NO	YES	NO	NO	NO	NO
SPORULATION	NO	NO	NO	NO	NO	YES

Table 3.10   The behaviour of 'normal' heterogeneous Rm 5 cells in the 15l
batch fermenter under different environmental conditions
(temperature, 30° C ;   incident light intensity, 9000 lux;
stirrer speed, 100 r.p.m.).

198.

(vii)   Standard 'run' P + S cells (PYR/MAL medium. No pH control).

A control experiment for P + S cells was performed with incubation
conditions identical to those used for 'normal' heterogeneous cells.
P+S cells from the culture reservoir were used as inoculum, and routine
medium (PYR/MAL and 0.1 $\underline{M}$ phosphate buffer, pH 6.8) was employed.
The cells were incubated anaerobically at 30° C with an incident light
intensity of 9000 lux and a stirrer speed of 100 r.p.m.

Characteristic behaviour of the culture was apparent, and a normal
growth curve obtained (Fig. 3.32). As was found with 'normal' heterogeneous
cells, there was correlation between the protein and bacteriochlorophyll
content of the culture. The growth efficiency of the P + S cells (stationary
phase absorbance 4.4), however, was greater than previously observed
with 'normal' cells (stationary phase absorbance 3.6). The soluble CO_2
tension reached a maximum of 43% (v/v) during late exponential growth of the
culture before dropping slightly to a value of 38% (v/v) by stationary phase. The
P+S cells appeared therefore to either produce more CO_2 or to utilise less
than 'normal' cells. This observation, however, was an artefact caused by
the existence of pH differences between the cultures. An equilibrium
exists between soluble CO_2 and bicarbonate ions in solution ($CO_2 \rightleftharpoons HCO_3^-$).
At low pH the equilibrium is displaced to the left, whereas at high pH it
moves to the right. Cultures of 'normal' heterogeneous cells always reach
a higher pH (~ 8.1) than that of P+ S cultures (~ 7.6), and this is
responsible for the apparent differences in the CO_2 tension during growth of
the two culture types, $\underline{i.e.}$ the drop in CO_2 tension exhibited at high pH by a
'normal' culture is not due to the utilization of CO_2 by the cells but to a
shift in the HCO_3^-/CO_2 equilibrium. By adding acid to the culture externally
and reducing the pH, it was possible to artificially increase the soluble CO_2
tension of a 'normal' culture to the corresponding level of a P + S culture.
Further proof of this shift in equilibrium was obtained by growing 'normal'
heterogeneous $\underline{Rm5}$ cells at constant pH, (run (ii)) whereby growth of the
cells in late exponential phase did not give rise to a drop in the soluble CO_2
tension.   The production of CO_2 during the growth of 'normal' heterogeneous
and P +S $\underline{Rm\,5}$ cells is very similar. The uptake of CO_2 by the two cell
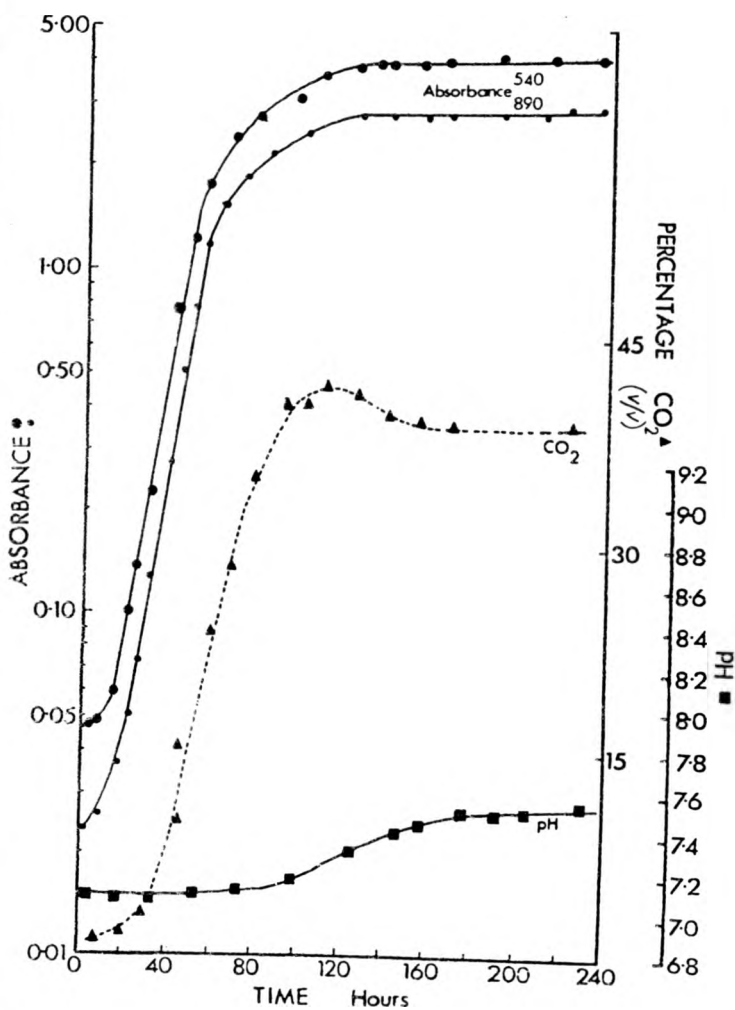types (P + S and 'normal') was investigated using $^{14}CO_2$.

Fig. 3.32

Properties exhibited by a batch culture of Rm 5 P + S cells grown in the
15 litre fermenter on PYR/MAL medium under routine conditions (30° C
9000 lux, 100 r.p.m.).

(●) Culture absorbance, 540 nm.   (■)   Culture pH.

(•) Culture absorbance, 890 nm.   (▲) CO_2 tension in suspension.

CO_2 uptake. P + S cells versus 'normal' heterogeneous Rm 5

The carbon dioxide uptake rates of P + S cells and 'normal' cells were compared by growth on routine medium (PYR/MAL and 0.1 $\underline{M}$ phosphate buffer, pH 6.8) under identical conditions in the presence of ^{14}C labelled carbon dioxide. 1 litre aspirators (see Section 2) were used as culture vessels and the cells incubated at 30° C with incident illumination of 2000 lux.

The CO_2 uptake by the P + S cells was indistinguishable from that of the normal cells (Figs. 3.33 and 3.34). In both cases there was good correlation between the growth of the cells (measured by absorbance at 540 nm) and CO_2 uptake. There was no evidence to suggest that the drop in the soluble CO_2 tension in the culture medium of 'normal' cells is a direct result of stimulated CO_2 uptake. CO_2 production and uptake by 'normal' heterogeneous cells appears no different from that of P + S cells.

Fig. 3.33

Uptake of $^{14}CO_2$ by a culture of P + S cells growing anaerobically on PYR/MAL medium at 30° C with an incident illumination of 2000 lux.
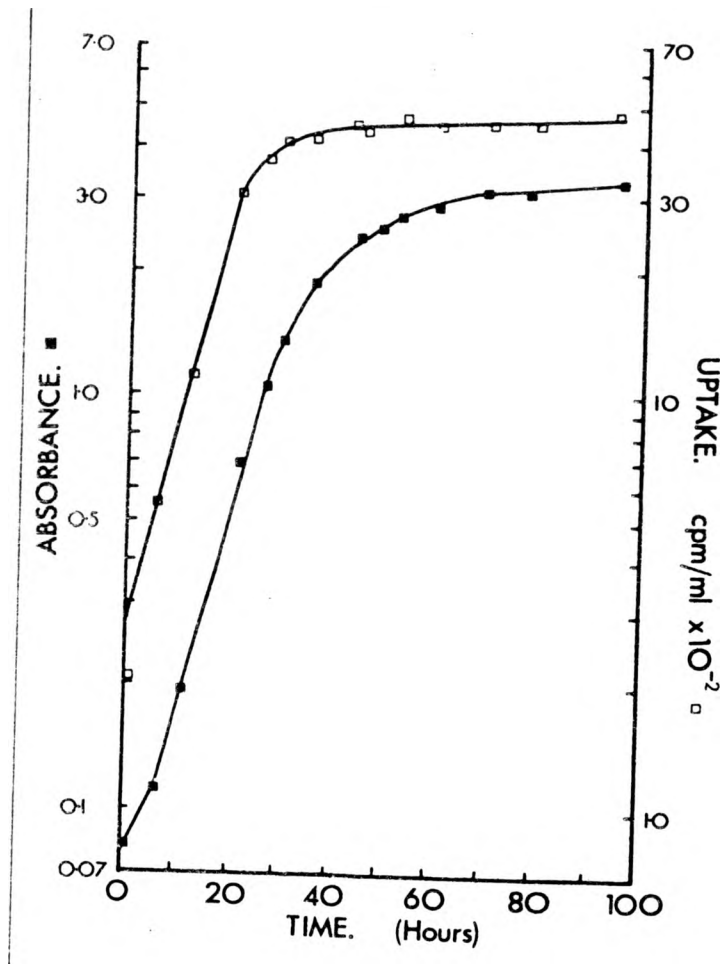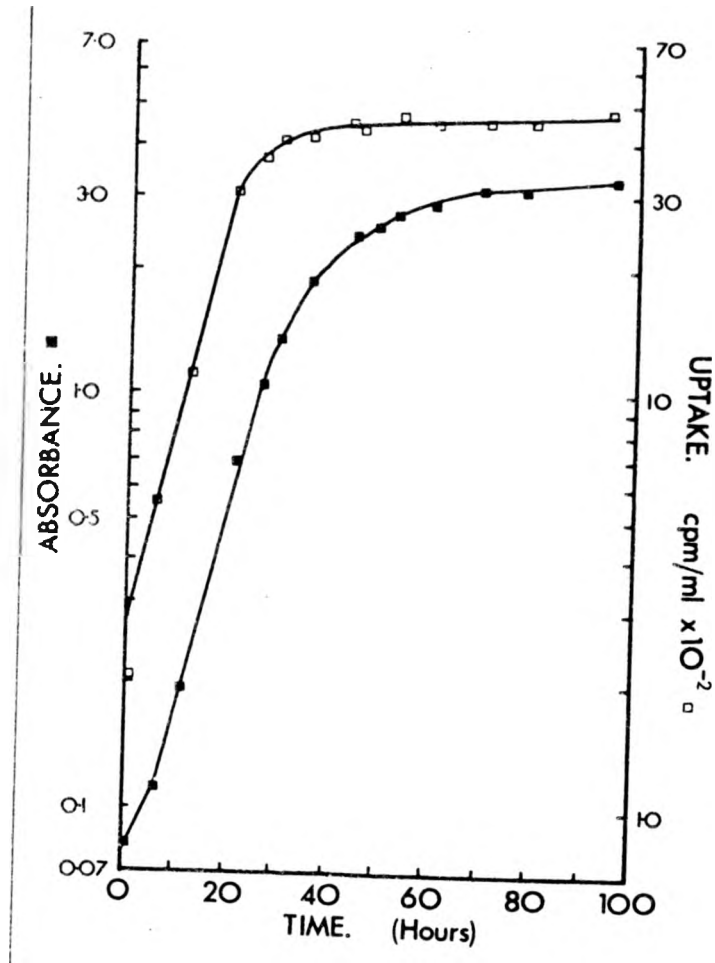
Fig. 3.33

Uptake of $^{14}CO_2$ by a culture of P + S cells growing anaerobically on PYR/MAL medium at 30° C with an incident illumination of 2000 lux.
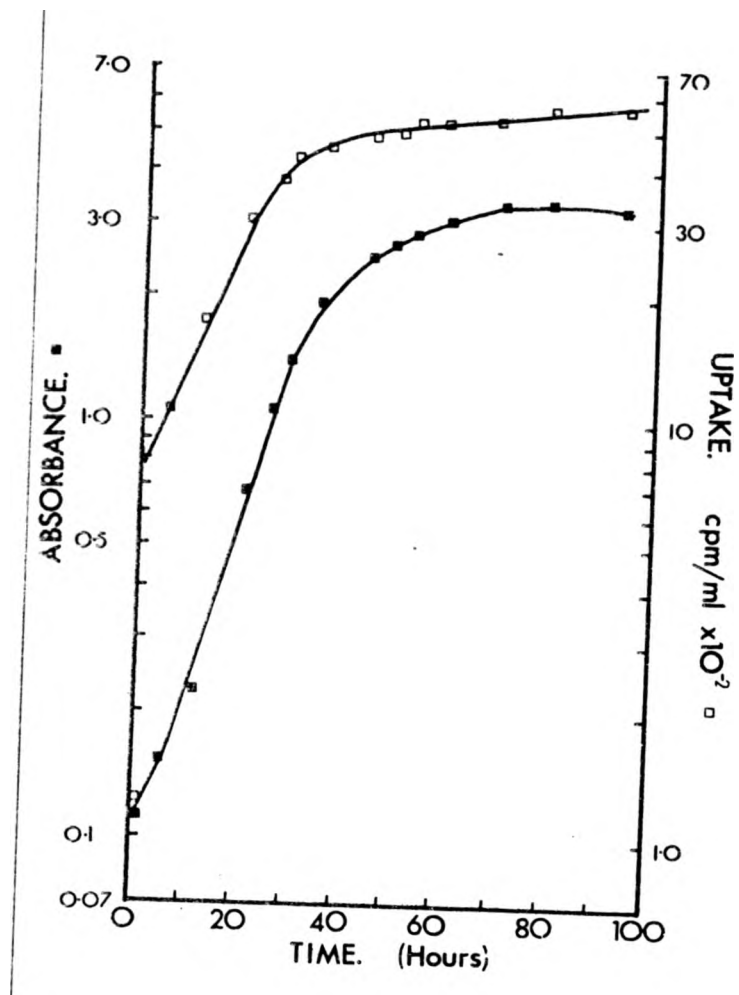
Fig. 3.34

Uptake of $^{14}CO_2$ by a culture of 'normal' heterogeneous Rm 5 cells growing anaerobically on PYR/MAL medium at 30° C with an incident illumination of 2000 lux.

(viii) P + S cells. Batch culture on PYR/high MAL medium.

P + S cells were cultured anaerobically (30° C, 9000 lux, 100 r.p.m.) on PYR/MAL growth medium and 0.1 M phosphate buffer, pH 6.8 which contained a high concentration of sodium hydrogen malate (sodium pyruvate, 1.0 g/l; sodium hydrogen malate, 2.5 g/l). Behaviour of the culture was abnormal, and not as would be predicted.

The pH response and CO_2 tension of the culture were fairly typical, (Fig. 3.35a), but due to the increased carbon concentration of the culture medium cell growth proceeded to high density (Fig. 3.35b). Stability of the P + S cells was unaffected and no rough colony types (characteristic of 'normal' cells) were detected when samples were plated out on to solid medium. During the late exponential phase of growth, however, cells of unusual phenotype appeared in large numbers. Such cells were very small, (Plate 3.40), angular, and often attached in pairs (Plates 3.41 and 3.42). Initially it was thought that they were small exospores. However, they bear little resemblance, either in shape, size or properties to the exospores formed by normal cells (Plates 3.40, 3.42, 3.43) which are surrounded by capsular material, highly refractile and produced by mother cells in multiples of up to 4. Unlike exospores which are very heat resistant and able to survive temperatures of 100° C for several minutes, the mini-'spores' are heat-sensitive and have a tolerance similar to vegetative cells, being unable to withstand a temperature of 60° C. Mini-'spores' show a greater likeness to constricted vegetative cells (Dow, 1974) which are formed infrequently by the normal budding process.
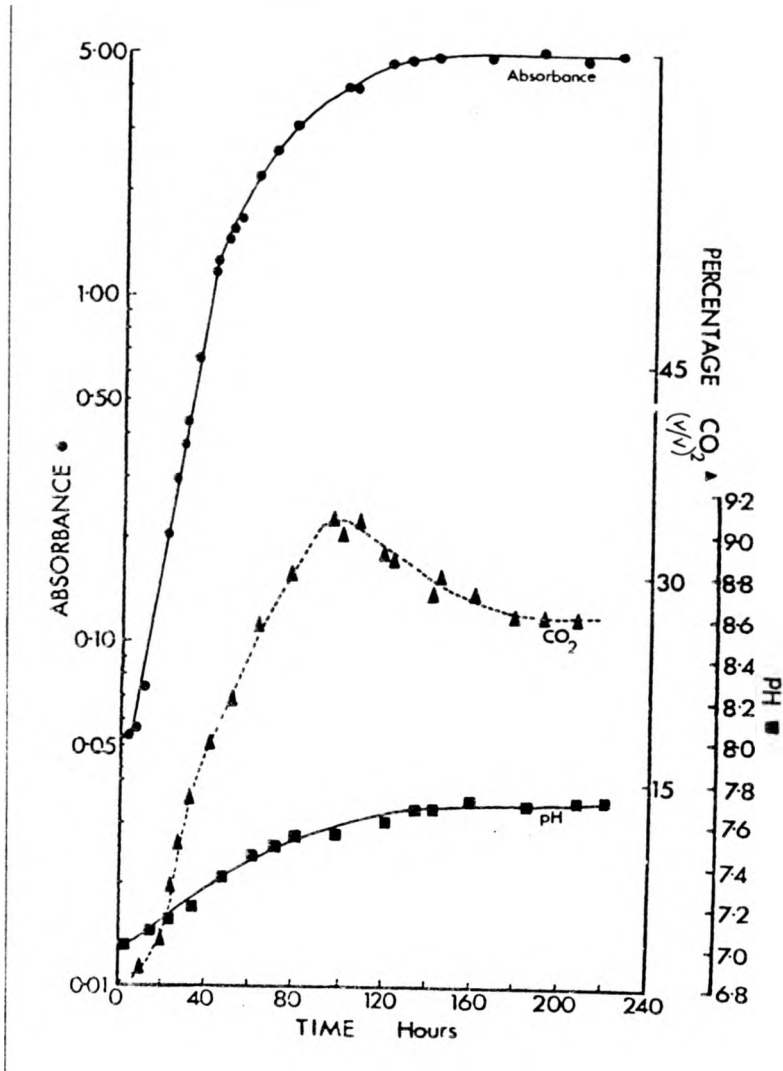
Fig. 3.35a

Properties exhibited by a batch culture of <u>Rm 5</u> cells grown in the
15 litre fermenter on PYR/high MAL medium $(30^{\circ}$ C, 9000 lux, 100 r.p.m.).

(●) Culture density. Absorbance 540 nm.

(▲) CO_2 tension in suspension.
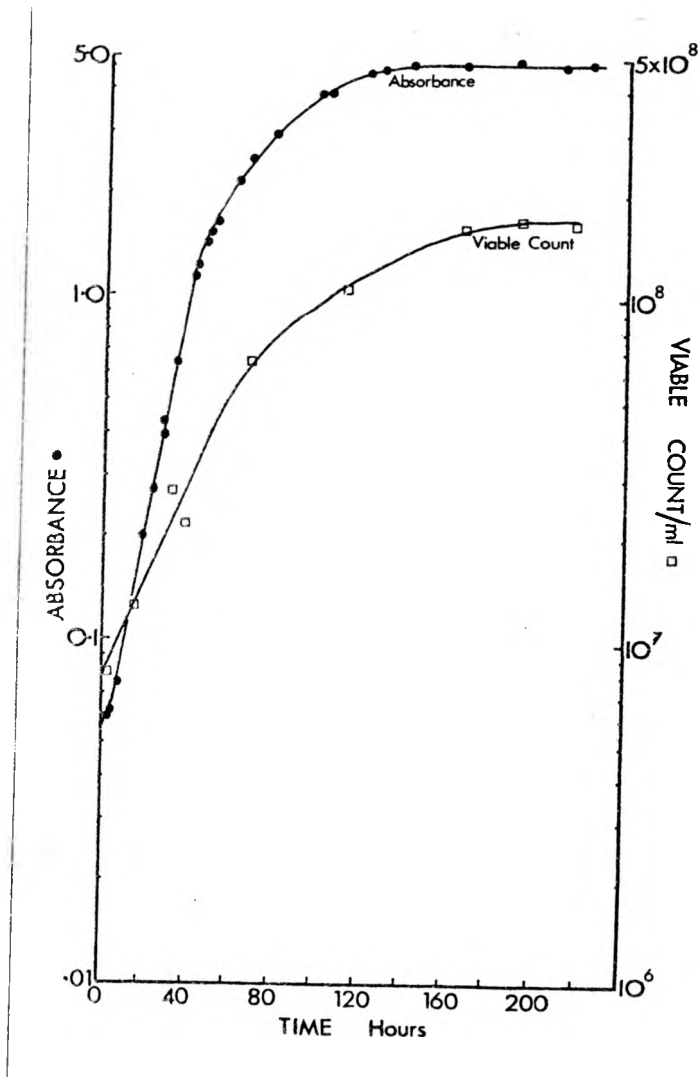
(■) Culture pH.

Fig. 3.35b

(●) Culture density. Absorbance 540 nm.

(□) Cell viable count per ml.
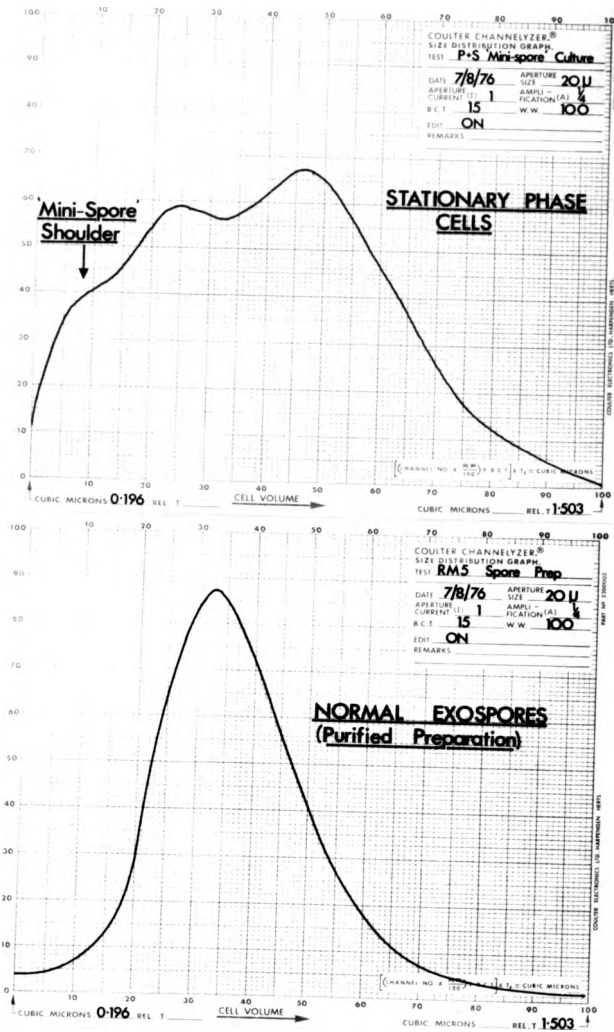
Plate 3.40

'Coulter profiles'

(A)   Stationary phase P + S cells (220 h   ).

(B)   Purified exospore preparation.
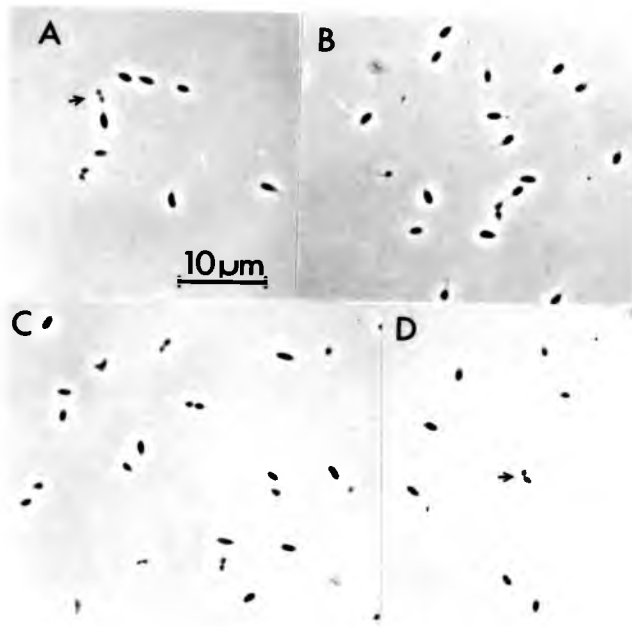
Plate 3.41

Phase contrast photomicrograph showing the small angular cells
produced by P + S cells during late exponential growth on PYR/high MAL
medium.

Plate 3.42

(Bars = 1 μm)
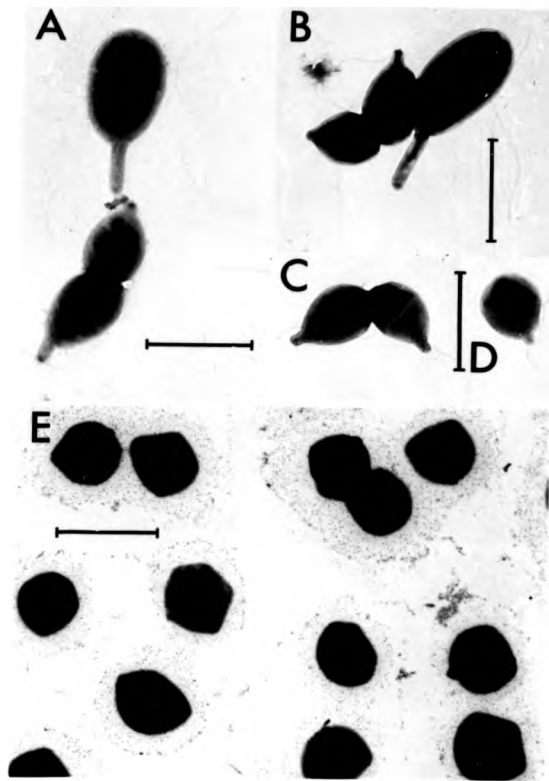
Electron micrographs   (gold palladium shadows)

(A)(B)(C)   Pairs of mini-'spores'.

(D)   Single mini-'spore'.

(E)   Exospores surrounded by capsular material produced by
             'normal' heterogeneous Rm 5 cells.
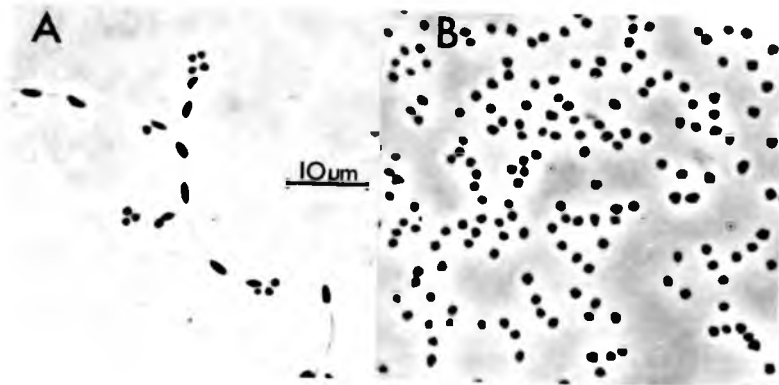
Plate 3.43

Phase contrast photomicrographs

(A)   Exospores attached to mother cells in multiples of 3 or 4.

(B)   Purified exospore preparation.

(ix)   P + S cells. Batch culture on sodium hydrogen malate (MAL) medium

P + S cells were allowed to grow on normal salts medium and 0.1 M phosphate buffer, pH 6.8, containing sodium hydrogen malate (1.5 g/l) as sole carbon source. The usual anaerobic growth conditions were employed $(30^{\circ}C, 9000$ lux, 100 r.p.m.).

Behaviour of the culture was unaffected by the change in medium composition. Stability of the P + S cells appeared normal since no cell growth on the sides of the fermenter was evident, and no multicellular arrays were observed in suspension. When plated out on to solid medium, no rough colony types were observed. As was the case with the corresponding experiment ('run'(iii)) involving 'normal' heterogeneous cells, the maximum culture density was carbon limited (Fig. 3.36a). Growth on medium in the absence of sodium pyruvate brought the expected reduction in the soluble CO_2 tension, and an increase in the stationary phase pH of the culture. Characteristic P+S 'Coulter profiles' (Plate 3.44) were evident throughout culture growth, and the size of the cells decreased considerably with culture age (Fig. 3.36b). However, since the size of P + S cells in continuous culture appeared to be little affected by growth rate, the reason for volume change under batch conditions is open to speculation.

Fig. 3.36a

Properties exhibited by a batch culture of Rm 5   P + S cells grown in
the 15 litre fermenter on MAL medium. (30° C, 9000 lux, 100 r.p.m.).

(•)   Culture density. Absorbance 540 nm.

(▲) CO_2 tension in suspension.

(■)   Culture pH.

Fig. 3.36b

(●)   Culture density. Absorbance 540 nm.

(○)   Total cell count per ml. (Coulter counter).

(△)   Mean swarm cell colume, μm^3. (Coulter channelyser).
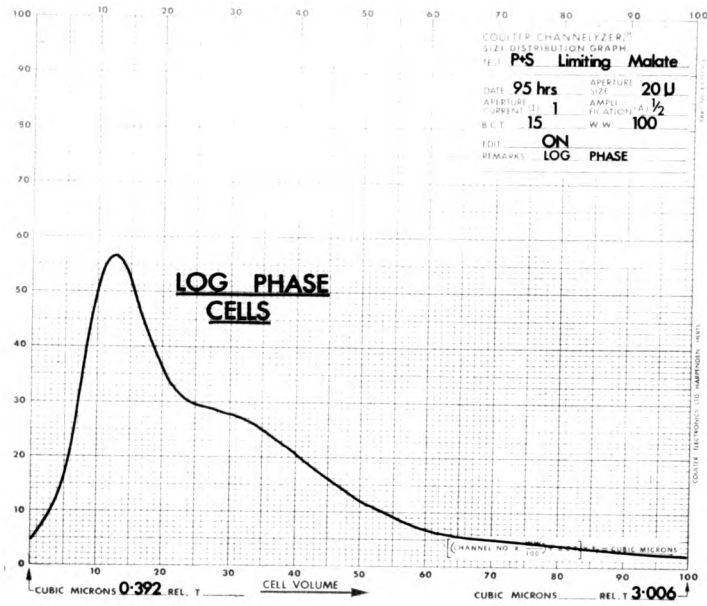
Plate 3.44

The 'Coulter profile' of P + S cells from the exponential growth
phase (95 h).

(x) P + S cells. Batch culture on sodium hydrogen malate (MAL) medium. CO_2 tension reduced.

The behaviour of P + S cells under conditions of very low carbon dioxide tension was studied. The culture was allowed to become established on MAL growth medium and 0.1 M phosphate buffer (pH 6.8) under the usual anaerobic conditions (30° C, 9000 lux, 100 r.p.m.) before oxygen free nitrogen was sparged through the suspension at a flow rate of 60 ml/min. A considerable reduction in the CO_2 tension resulted, accompanied by a rapid increase in the pH of the culture (Fig. 3.37a). Significant limitation of culture growth by the high pH was not apparent, and behaviour of the culture seemed little different from the previous experiment. 'Coulter profiles' (Plate 3.45) characteristic of a typical P + S culture were once again obtained throughout growth. However, the decrease in cellular volume was more rapid and extreme than usual, and cells in the stationary phase culture were unusually small (mean volume 0.44 μm^3)(Fig. 3.37b). A few mini-'spores' were observed by the late exponential phase of culture growth.

Once again there was no evidence to suggest that the stability of the P+S cells had been affected, and the appearance of multicellular arrays did not occur. Towards the end of the experiment, samples were plated out on to solid medium and incubated in anaerobic bags. No rough colony types were detected.
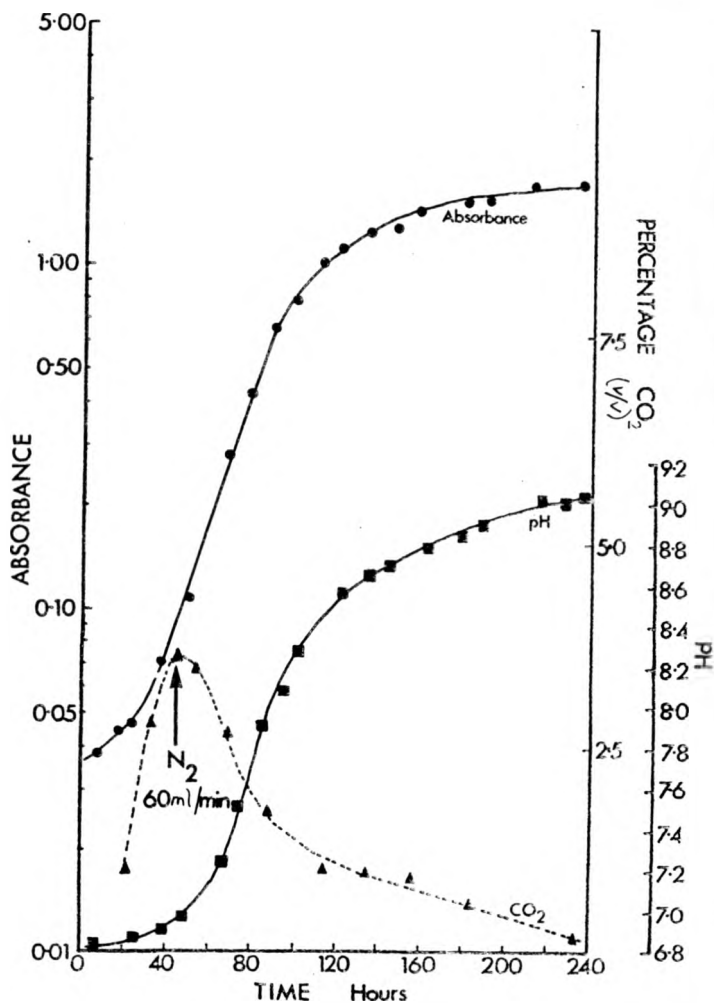
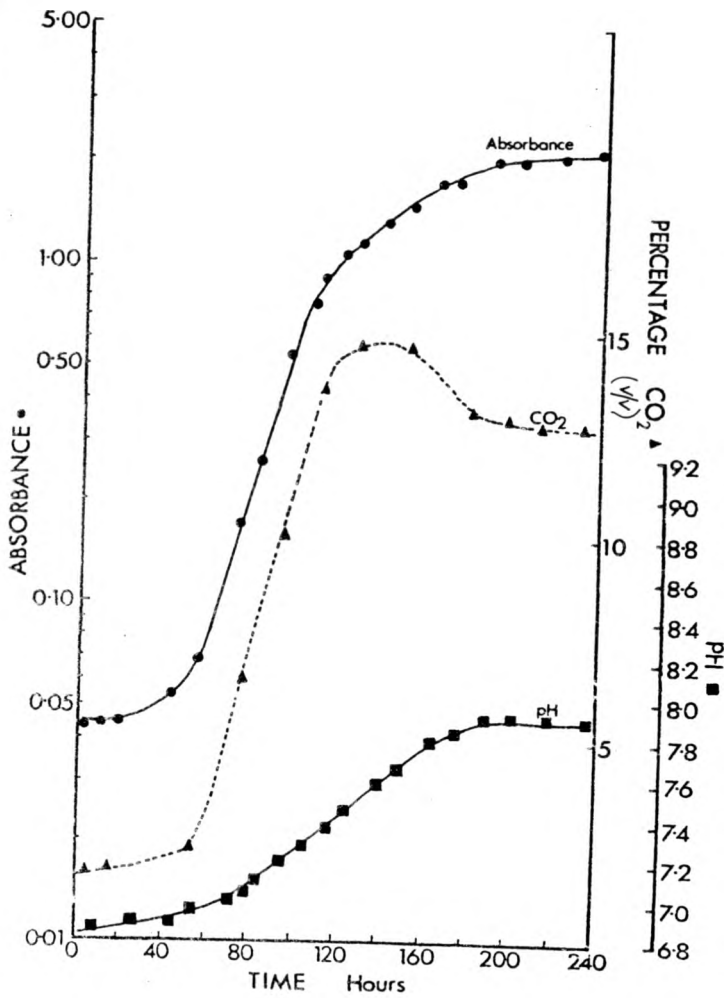Fig. 3.37a

Properties exhibited by a batch culture of Rm 5 P + S cells grown in
the 15 litre fermenter on MAL medium under conditions of reduced CO_2
tension. (N_2 sweep 60 ml/min). (30° C, 9000 lux, 100 r.p.m.).

(●) Culture density.

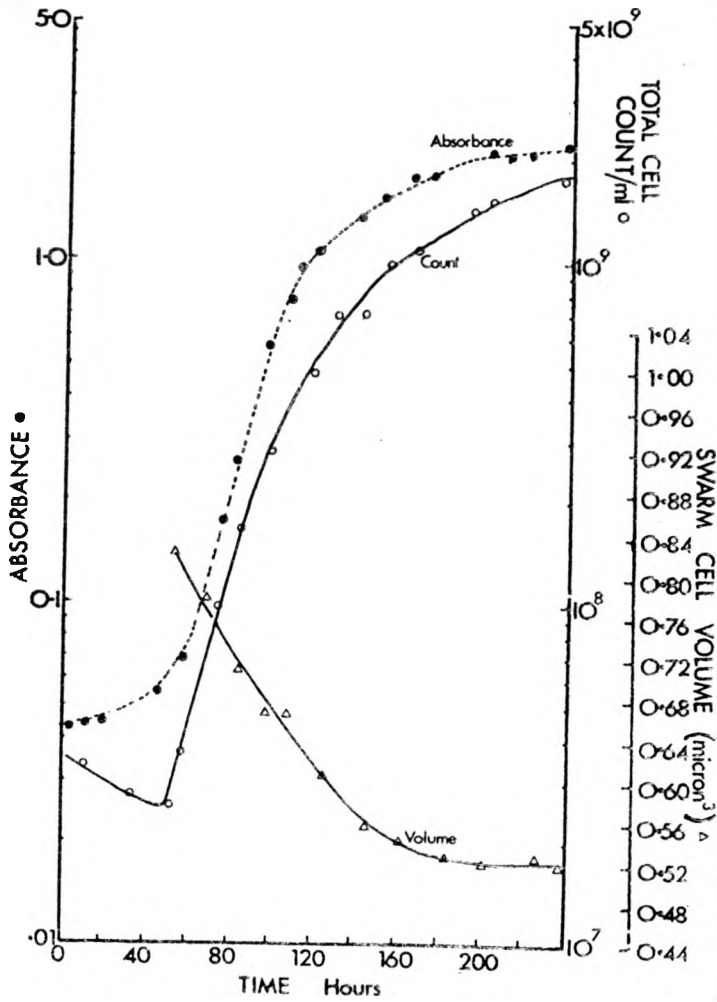(▲) CO_2 tension in suspension.
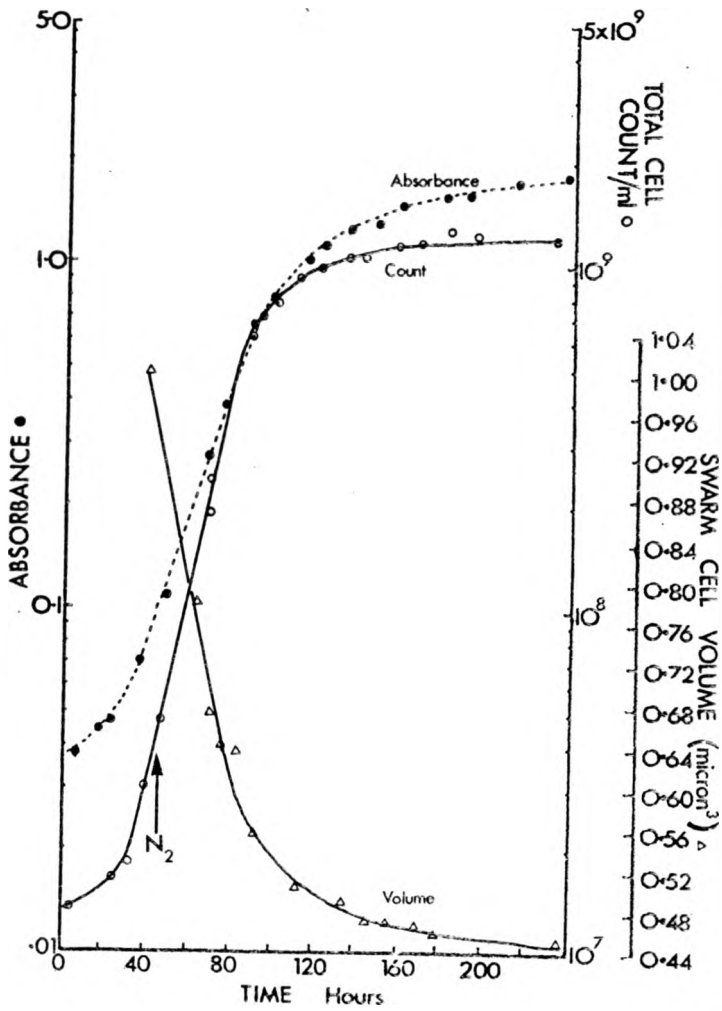
(■) Culture pH.

Fig. 3.37b

(●) Culture density. Absorbance 540 nm.

(○) Total cell count per ml. (Coulter counter).

(△) Mean swarm cell volume, μm^3. (Coulter channelyser).
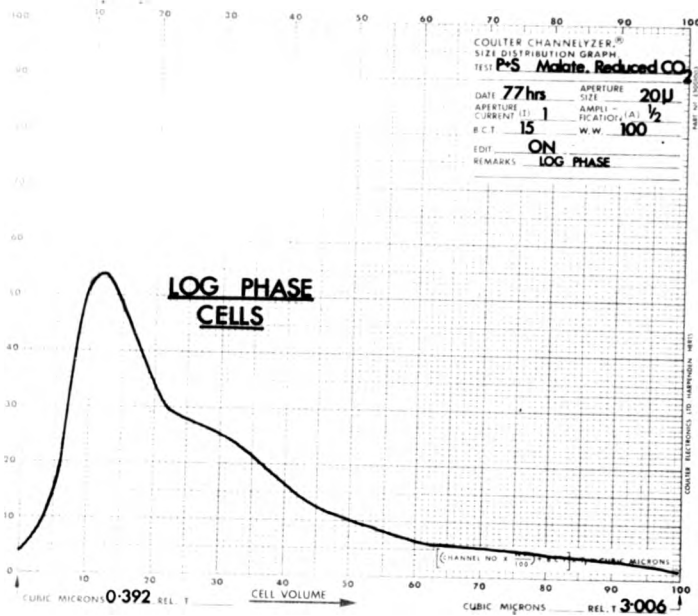
Plate 3.45

The 'Coulter profile' of P + S cells from the exponential growth
phase (77 h   ).

Summary of the behaviour of Rm 5 P+ S cells in the 15 litre batch
fermenter experiments

The major features concerning the behaviour of P + S cells in the
15 litre batch fermenter under different environmental conditions are
displayed in Table 3.11. Stability of P + S cells was unaffected by growth
under any of the conditions employed and the appearance of multicellular
arrays was not evident. The growth rates of the simplified cells were
comparable to the 'normal' heterogeneous Rm5 cells, but the growth
efficiency of the former was higher. Higher values of soluble CO_2 tension
were evident, but this was a reflection of the growth of the P + S cells
having a less marked effect on the culture pH than normal cells (and the
consequent shift in the $CO_2 \rightleftharpoons HCO_3^-$ equilibrium).

Growth of P + S cells on medium containing high carbon concentration
gave rise to the appearance of small angular cells, resembling exospores
but devoid of their characteristic properties. The nature and function of
these cells has yet to be investigated.

Characteristic and similar 'Coulter profiles' of P + S cells were
apparent under all conditions studied. The size of the cells, however,
decreased rapidly with culture age.

	RUN (vii) PYR/MAL	RUN (viii) PYR/high MAL	RUN (ix) MAL	RUN (x) MAL N_2 60 ml/min
DOUBLING TIME (h)	7.4	7.0	12.9	15.0
STATIONARY PHASE ABSORBANCE	4.4	5.0	2.2	1.9
STATIONARY PHASE pH	7.55	7.7	7.9	9.5
PERCENTAGE CO_2 MAX (v/v)	43.5	35.0	14.9	3.1
PERCENTAGE CO_2 FINAL (v/v)	33.8	26.0	12.7	0.0
PERCENTAGE NORMAL SWARM CELLS	0	0	0	0
CULTURE BEHAVIOUR	Typical	Atypical mini-cell production	Typical	Typical

Table 3.11 The behaviour of Rm5 P + S cells in the 15l batch fermenter under different environmental conditions (temperature 30° C ; incident light intensity, 9000 lux; stirrer speed, 100 r.p.m.).

Conclusion

The prime objective of this project was an extensive study of morphogenesis and differentiation in Rhodomicrobium Rm5 , in order to evaluate the potential of this organism as a model microbial differentiation system. The initial work, performed by Dow (1974) was directed towards a greater understanding of the processes involved in the obligate differentiation of this organism, i.e. swarm cell maturation, tube, bud and daughter formation. In contrast, I decided to pursue the inducible differentiation patterns associated with Rm5 , with a view to confirming earlier suggestions (Dow, 1974) that the environment has profound effects on the phenotypic expression of this organism. Once such a relationship had been established, it was hoped to identify the environmental factors associated with the induction of swarm cells and exospores.

Since the use of batch culture means that the cells are subject to a continually changing environment (light, pH, nutrient concentration), the use of continuous culture to study the effect of different defined conditions on Rm5 morphology and composition was favoured. The growth of a 'normal' heterogeneous population of Rm5 cells in a chemostat under relatively low dilution rates led to a complete change in culture composition. The characteristic multicellular arrays were lost, to yield a steady state composed entirely of a previously undocumented cell type (P + S cells), possessing a simplified life cycle. Growth of the cells in this cycle eliminated the major problem, that of extensive wall growth on the sides of the fermenter vessel normally associated with growth as multicellular arrays. By variation of the cultural conditions (dilution rate, CO_2 tension) it was possible to alter the type of cellular expression displayed by Rm5 and perpetrate the reappearance of multicellular arrays. Such a change, however, was accompanied by the seemingly insurmountable problem of wall growth. Consequently it was concluded that although the chemostat was ideal as a 'culture reservoir' for simplified cells, its use as a tool for studying the environmental influence on cellular exrpession in Rm5 was considerably restricted.

A new approach was adopted, and a large scale batch fermenter was modified, enabling the growth of Rm5 under various, but carefully monitored conditions to be closely observed, with the aim of correlating cellular expression with transient environmental changes. Swarm cells separated from the late exponential phase of a 'normal' heterogeneous culture, grown in this system were found to give rise to 'simplified' cells. The expression of swarm cells in this manner was found to be associated with the time of maximum CO_2 tension in the culture. Although the possibility that 'simplified' cells may be mutants has not been eradicated, it would seem unlikely, and the evidence to date, employing batch and continuous culture techniques would suggest that such cells are an inducible form of expression which may be produced as a response to conditions of high CO_2 tension. The very existence of 'simplified' cells has led to the conclusion, contrary to previous beliefs, that microcolony formation is not obligatory for swarm cell synthesis.

Other preliminary investigations, using the batch fermenter system, confirm the importance of the environment in regulating cellular expression in Rm5. Light intensity, pH and CO_2 tension are involved with swarm cell production and maturation, whereas the presence of trace elements was found to be a requirement for successful sporulation.

Potential

In terms of overall complexity, Rhodomicrobium vannielii (Rm 5) is unique. It possesses a polymorphic life cycle undergoing obligate well defined morphogenesis. In addition, changes in cellular expression (e.g. the 'simplified' vegetative life cycle) can be induced by alteration of environmental conditions (Figure 4.1). Rhodomicrobium has adopted obligate polar growth (budding) as a mode of cell growth in preference to the more widely observed intercalatory growth. Nevertheless, cell division in both situations is by binary fission. Symmetrical cell division of monomorphic bacteria, i.e. E.coli, results in the formation of two qualitatively and quantitatively identical siblings. In complete contrast, one of the most significant features of obligate polar growth is the asymmetry of cell division with the formation of two cells differing both quantitatively and qualitatively, i.e. they each have differing ages and are at different stages of cell development. The immature daughter cell has a transitory existence and has to undergo obligate differentiation to become a mature mother cell capable of reproduction. Such daughter cells are unique in so far as a series of morphological and molecular events occur which are never again repeated during the existence of that particular cell. In contrast, the stalked mother cell is structurally unaltered, undergoing only a small amount of filament synthesis before initiating new daughter cell formation.

The fact that large scale synchronous cultures of Rm 5 swarm cells can easily be obtained enables the pattern(s) of synthesis of enzymes and other proteins to be followed during the obligate differentiation of a swarm cell into a mature mother cell. Comparison of protein fractions can be effected by two dimensional gel electrophoresis, allowing direct comparison and identification of cellular components appearing and disappearing during the cycle.

The potential of Rm 5 as a model system for exploring differentiation and morphogenesis is immeasurable and is believed to surpass others being currently investigated. Considerable attention has now been focussed on obligate differentiation in the Caulobacter system, whereas environmentally stimulated sporulation in the Bacillus system has always been widely researched.

These are two unrelated systems, whereas the features of obligate and inducible differentiation exhibited by these two organisms are prevalent in a single species - Rhodomicrobium. Many of the simpler features and properties of this organism have been elucidated, and the next step is obviously to investigate the co-ordination and control of cellular expression in this organism at the molecular level. The results presented in this thesis suggest that vegetative Rm5 cells possess the knowledge to express themselves in any of at least 4 different ways.

(i) A vegetative mother cell can give rise to vegetative daughter cells which remain linked to the mother cell but are functionally separated by cross wall plugs.

(ii) It can give rise to 'normal' swarm cells which develop into 'normal' mother cells, i.e. capable of forming multicellular arrays.

(iii) It can give rise to 'simplified' swarm cells which form P + S cultures.

(iv) It can give rise to angular heat resistant exospores.

All these reproductive processes involve the formation of daughter cells at some distance from the main body of the 'mother' cell, where one can assume cellular activities are centred. The most complex situation is the formation of daughter cells in branched sequences. Proposing a single basic model which satisfies all the morphological variations seen in this organism must take into account large numbers of fundamental problems not encountered by a simple bacterium such as E. coli. Such a concept has been discussed by Whittenbury and Dow (1977) and a model proposed which appears to satisfy most of the (morphological) requirements of Rhodo-microbium.

Probably the most pertinent questions to ask concern the control and co-ordination of differentiation and morphogenesis in this organism, e.g.

(i) How does the cell decide which form of expression to adopt ?

(ii) When does the cell decide which form of expression to adopt ? Are some cells preconditioned ?

(iii) When the decision is made, how is it effected ?.

It would appear that sufficient 'groundwork' has now been completed to enable these problems to be tackled. Results suggest that environmental

MATURATION

(OBLIGATE
          DIFFERENTIATION)

ENVIRONMENTALLY INDUCED DIFFERENTIATION

Simplified cells

Multicellular arrays

Exospores
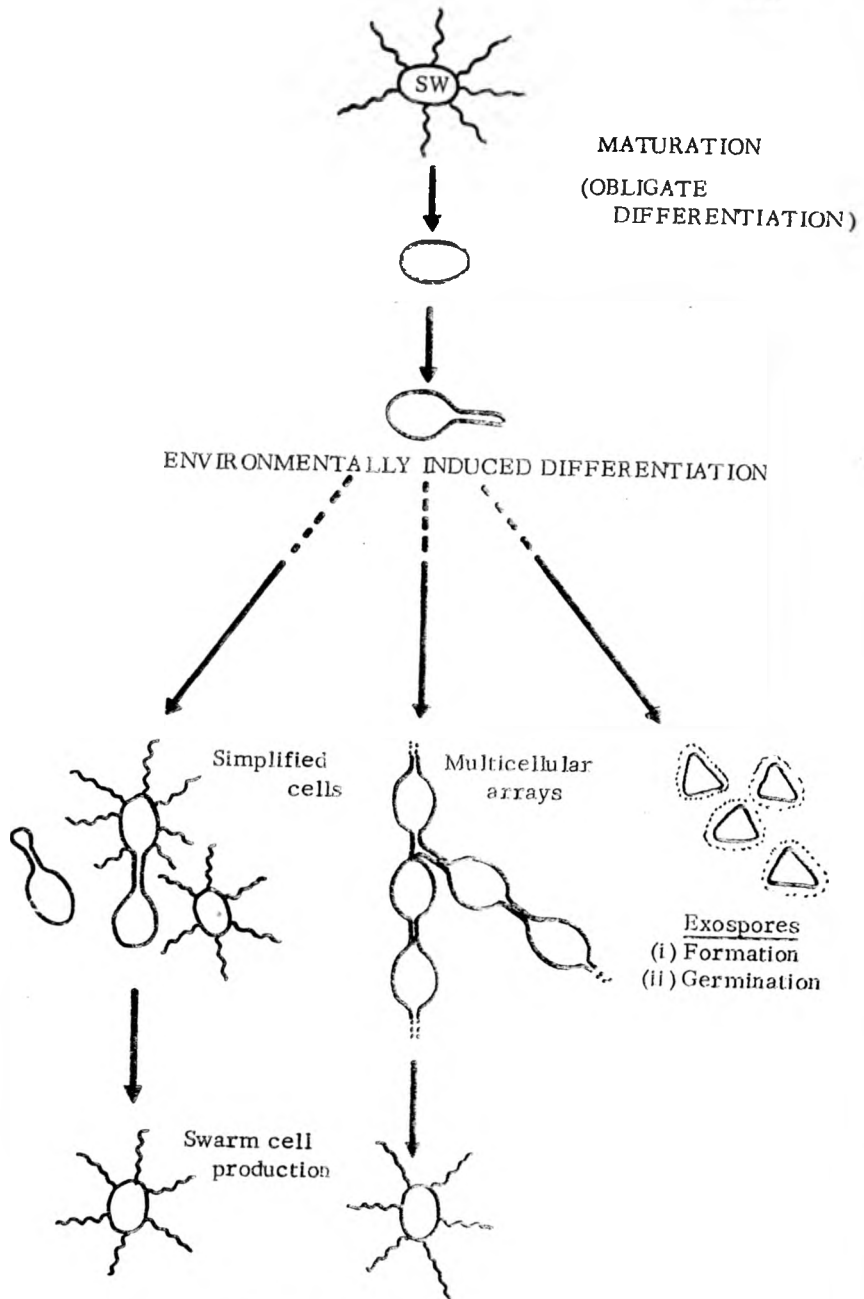(i) Formation
(ii) Germination

Swarm cell production

Fig. 4.1

Diagrammatic representation of obligate and environmentally induced changes in cellular expression displayed by Rhodomicrobium Rm 5.

factors (light, pH, CO_2) are involved in the control of cellular expression in Rhodomicrobium.   Further variations of environmental conditions, using the 15 litre batch fermenter, should enable these environmental stimuli to be 'pinpointed'. It would also be interesting to closely monitor aerobic dark growth of Rhodomicrobium using the 15 litre batch fermenter.

Control of sequential cell changes at the level of transcription and translation have been postulated in both Bacillus and Caulobacter. Rhodomicrobium Rm 5 now presents another system more diverse than the two aforementioned in which direct analysis of these control mechanisms is feasible. The isolation and puficiation of DNA dependent RNA polymerases, and ribosomal proteins, from the different Rm 5 cell types, and their comparison by two dimensional gel electrophoresis presents few problems. The sensitivity and resolution of the gel system should allow even the slightest modifications to be observed.

Rm 5 would also provide a very suitable system to investigate the involvement of cyclic nucleotides in the control of differentiation since nucleotide levels could be correlated with easily recognizable developmental stages.   The cyclic nucleotide levels in the different cell types could be measured and compared, and it would be interesting to see if a culture of 'simplified' Rm 5 could be made to grow 'normally' by the external manipulation of cyclic nucleotide levels in the culture medium.

Obviously the experiments one could peform with an organism of this type are limitless. It would be comforting to think that the results obtained so far, and all future knowledge using this organism, can ultimately be extrapolated to give a fuller understanding of the differentiation and development processes of higher organisms.

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