RHODOPSEUDOMONAS PALUSTRIS: A MODEL OF BACTERIAL DIFFERENTIATION

by

Donald Westmacott, B.Sc.(Bath)

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In loving memory of my dear Mother and Father.

DECLARATION

I hereby declare that this thesis has been composed by myself, and has not been used in any previous application for a degree. The work of which it is a record has been carried out by myself and all sources of information have been specifically acknowledged by means of references.

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SUMMARY

Differentiation in the budding photosynthetic bacterium Rhodopseudomonas palustris was studied both in its own right and in the context of other comparable bacteria; particularly R. acidophila and Rhodomicrobium vannielii. Together, these three Rhodospirillaceae present a gradient of morphogenetic complexity ideally suited to differentiation studies.

Semi-defined conditions were determined which gave consistent morphology and fast growth rates. Heterogeneous cultures of R. palustris were then synchronised by selecting swarmer cells by sucrose gradient sedimentation. Morphology and ultrastructure of the cell division cycle exhibited by R. palustris were examined in some detail and compared with R. acidophila. Both organisms exhibited differentiation at the sub-cellular level and by dividing to give two dissimilar cells. The distribution of cell types, stepwise doublings, particle volume distributions, optical density changes, cell motility, protein and DNA synthesis and probably also RNA synthesis were all examined during synchronous growth of R. palustris. Penicillin treatment showed that cell growth was by polar, unidirectional synthesis of wall material. Nalidixic acid, which inhibits DNA replication, modified the cell development by inhibiting cell division and giving rise to abnormal cell elongation. Studies with nalidixic acid also confirmed that there was periodic DNA synthesis during the normal cell cycle. Observations on the location of cell division in elongated cells after removal of the nalidixic acid, considered alongside the polar unidirectional mode of cell growth, allowed a model correlating chromosome replication and cell envelope growth to be proposed.

Some preliminary investigations were made of mutants of R. palustris. Temperature-sensitive mutants were obtained, but these did

not appear to be blocked at any particular stage of development. A phage specific for one strain of R. palustris was investigated and appeared to be temperature-sensitive in some step of its growth cycle. This might permit its use for transduction. Both temperature-sensitive mutants and a method of genetic transfer were considered invaluable for further studies of the control of R. palustris differentiation.

GENERAL INTRODUCTION

The biochemistry of differentiation and its control in higher organisms has proved to be a particularly difficult field for the biologist largely as a result of the fact that many of the biochemical aspects of such organisms at the cellular level are still poorly understood. An understanding of these processes of differentiation is particularly relevant to numerous aspects of medical physiology: spermatogenesis, oogenesis, embryonic origins and tumor tissue growth to name but a few.

The term differentiation has been used in many different senses in biology but perhaps the most useful interpretation would encompass all these applications of the term. Differentiation may, therefore, be considered as any situation where meristematic cells give rise to two or more types of cells, tissues or organs which are qualitatively different from each other (Waring, 1971). Not only would such an interpretation include the control aspects and biochemical and organelle changes within the cell cycle but also cell growth and multiplication and ultimately intercellular communication and spacial arrangement of differentiated cells. Such an interpretation is considerably more useful than the extremely restrictive approach of Wright (1967) who considers differentiation and morphogenesis only to occur under starvation conditions as an endogenous, self-sufficient mechanism.

Because of the greater knowledge of the biochemistry and genetics and the simplicity of culture of lower organisms workers have often examined differentiation in such organisms with the intention of gaining information to be extrapolated to higher organisms (Shapiro, Agabian-Keshishian & Bendis, 1971; Donachie, Jones & Teather, 1973; Keynan, 1973; Garrod & Ashworth, 1973). Such intentions should and are usually expressed with some degree of caution, particularly when comparing prokaryotes with higher organisms. Indeed, some consider studies of differentiation in lower organisms to be most valid simply

in giving a greater understanding of processes in these organisms *per se* (Bonner, 1973). Such an approach then permits the consideration of trends amongst the spectrum of organisms, indicating basic concepts of development.

Models of differentiation amongst the simple eukaryotes have been successfully studied by a number of workers. Although in the more complex examples these are relatively closely related to higher organisms, some are on a very simple scale. Perhaps the most simple studies have been by Mitchison and his colleagues on the cell cycle of *Schizosaccharomyces pombe* (Mitchison, 1973). Although the cell cycle of an organism is perhaps not the most obvious example of differentiation it does show morphogenesis, particularly at and about cell division and, as will be shortly noted, periodic syntheses which are the manifestation of ordered gene expression. Mitchison (1973) has described the appearance of *S. pombe* as a "scaled-up version of a bacterial rod" although cell elongation is by apical growth and cell physiology is typical of a eukaryote. The organism has proved easy to study in pure culture using defined media. Equally important for studies of the cell cycle, synchronisation of the cultures has been achieved both by sucrose gradient sedimentation (Mitchison & Vincent, 1965) and induction using deoxyadenosine treatment (Mitchison & Creanor, 1971a). Although chromosome mapping in *S. pombe* is not as well developed as in *Saccharomyces cereviseae* there is a good genetical background for cell cycle studies. However, particular attention has been paid to mutants in ascospore formation and the concomitant meiosis (first isolated by Bresch, Miller & Egal, 1968).

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and in addition DNA synthesis shown only to occur during a specific period of 10% of the cell cycle (Mitchison & Creanor, 1971b). When DNA synthesis was specifically inhibited the subsequent pattern of periodically synthesised enzymes was unchanged (Mitchison, 1973). This showed that the ordered stepwise appearance of enzymes was controlled by a means completely independent of the cycle of DNA replication and cell division. This conclusion was further substantiated by the fact that populations examined after induction synchronisation by temporarily blocking DNA synthesis revealed an approximately linear pattern of enzyme synthesis rather than the expected stepwise synthesis. This was interpreted as showing that the synthesis of enzymes was unaffected by the synchronisation procedure although cell division and DNA replication were synchronised (Mitchison, 1973).

Other patterns of enzyme synthesis have been demonstrated in S. pombe. Firstly, enzyme synthesis in a continuous, linear fashion has been shown to double in rate of synthesis at particular points in the cell cycle. Secondly, some enzymes have been shown to double in inducibility or "potential" (Kuempel, Masters & Pardee, 1965), also at specific times. As will be discussed later, similar doublings in enzyme patterns of prokaryotes can be regarded as due to gene dosage effects; however, in S. pombe DNA replication occurs only over a short period and quite separate from (about 0.35 of a cycle before) the timing of the enzyme changes. Mitchison & Creanor (1969) and other workers have detected chromosomal changes at about the same time as the enzyme increases. It has therefore been suggested that only at this time, the "critical point", is the genetic material made available for transcription by some means, perhaps analogous to histone action in higher eukaryotes. More recent results have, however, cast some doubt on such an explaination since if the timing of DNA synthesis is delayed sufficiently enzyme potential doubling can occur immediately (Mitchison, 1973). Indeed,

considerations of the control of the cell cycle in S. pombe are only just becoming understood, but it is probably true to say that this organism might prove as informative about eukaryotes as Escherichia coli has about prokaryotes.

Budding species of yeasts have been used as very informative examples of eukaryotic differentiation. Such studies have been in three fields: the biochemistry of morphogenesis, enzyme synthesis during vegetative growth and ascospore formation and germination.

Bartnicki-Garcia & McMurrough (1971) discussed the structure and composition of yeast cell walls as well as the pattern of wall construction as revealed by fluorescent stains, and by use of antibodies, autoradiography and electron microscopy. When they considered aspects of cell division it was pointed out that although the generation time of Sacc. cereviseae can vary from 1.3 to 15 h the budding phase is always 1.3 to 2 h long. It was suggested that budding might depend on accumulation of trigger compounds or metabolic pools to specific levels. However, it is interesting to note that DNA synthesis occurs in a stepwise fashion at bud emergence and perhaps this indicates some controlling influence of DNA replication on bud formation or vice versa. Bartnicki-Garcia & McMurrough (1971) also discussed environmental control of yeast division. Both Candida albicans and Mucor rouxii respond to a wide variety of environmental changes by changing between a mycelial elongated form and a typical budding yeast form. The biochemical basis for such changes is becoming particularly well understood in Candida albicans where the reductive breakage of cell wall disulfide links can cause a plasticising of the cell wall, favouring the yeast form of growth. Another pattern of yeast morphogenesis is displayed by Trigonopsis variabilis where there is an interchange between a curious triangular cell shape and the conventional yeast form. Although changes in

phospholipid have been implicated the causes of the shapes are not known.

Halvorson, Carter & Tauro (1971) have listed 28 enzymes which are synthesised periodically during the cell cycle of Sacc. cereviseae in a constant environment. In contrast to S. pombe, none were synthesised continuously but specific periods of synthesis were observed throughout the cycle. In order to explain the control of such enzyme synthesis Halvorson and his colleagues have proposed that genes are transcribed in the same order as their linear sequence on each chromosome. Various predictions of such models are fulfilled (Halvorson et al, 1971) and notably by the limited evidence that the order of four gene loci on a single chromosome is the same as the order of timing of their enzyme products in the cell cycle (Tauro, Halvorson & Epstein, 1968). Clearly such a model cannot be applied to the "critical point" observations in S. pombe, but those observations were only made when considering enzyme inducibility in the cycle which is thought to be a gene dosage problem. Periodic enzyme synthesis is presumably controlled separately and it remains to be seen if the order of appearance of periodic enzymes in S. pombe is the same as that of their respective gene loci on individual chromosomes. In this aspect the advanced state of mapping of chromosomes of Sacc. cereviseae in comparison with S. pombe has been a great advantage.

Studies on ascospore formation (reviewed by Tingle et al, 1973) and germination (Rousseau et al, 1972; Rousseau & Halvorson, 1973) have largely been carried out on Sacc. cereviseae. In contrast to differentiation in the cell cycle it is important to note that such spore formation is environmentally triggered rather than obligatory and as such offers a rather more obvious and clearly defined example of differentiation. Detailed electron microscopy studies of sporulating Sacc. cereviseae are gradually revealing the ultrastructural complexities of the process as a whole (Moens, 1971; Moens & Rapport, 1971a,b; Guth, Hashimoto &

Conti, 1972). Biochemical changes that occur during ascospore formation have also been considered alongside ultrastructural aspects (see review of Tingle et al, 1973). Perhaps yeast sporulation is most useful as a differentiation model in offering an induced process of meiotic division, for vegetative cells are diploid and ascospores haploid. It is clear that mutants blocked at different stages of meiosis might yield a wealth of information on a process of fundamental importance to higher eukaryotes and which cannot be studied in prokaryotes. Although a number of such mutants have been obtained (Esposito & Esposito, 1969; Esposito et al, 1970) genetic analysis of sporulation in yeast has presented several problems. Not least of these is the fact that such analysis is accomplished by studying meiotic products and therefore mutants blocked in meiosis cannot be characterised unless they are of a conditional nature.

Yeasts, therefore, do present some problems when studied as models of differentiation, however because of their uncomplicated nature and ease of culture, they may prove to be one of the most useful groups of eukaryotic organisms examined.

Differentiation has also been considered in filamentous fungi (Gooday, 1973; Smith & Anderson, 1973). Because of the complexity of the sexual and asexual life cycles of such organisms studies have rarely been at the level of molecular control, although one notable exception is the investigation of fungal sex hormones in Mucorales (Gooday, 1973, 1974). If the metabolite of β-carotene, trisporic acid, is added to an unmated culture of Mucor mucedo sporangiophore production is suppressed and the appearance of zygophores stimulated. In vivo, trisporic acid is known to be produced by both (+) and (-) strains during mating and gives rise to the initiation of physical contact and zygophore formation. As such, this simple diffusible chemical plays

a key role in the sexual process perhaps of all Mucorales. Although the biochemistry of the action of fungal sex hormones is not yet understood they do offer a simple system of cell-cell interaction which may be extrapolated to more complex systems.

A certain amount of interest has also been shown in the molecular aspects of apical growth of fungal hyphae. To date, information on hyphal growth has largely been ultrastuctural detail and autoradiographic and lytic patterns. However, Bartnicki-Garcia (1973) has collated such information and proposed a model for the mechanism of apical growth in fungi. Although there is a considerable lack of knowledge in this field, the physical dimensions of such polar growth may allow basic principles to be laid down in a way not possible with polarly growing prokaryotes.

Studies on differentiation in slime moulds appear particularly interesting. Investigations have largely been carried out with the cellular slime mould Dictyostelium discoideum. This work has recently been reviewed by Sussman & Sussman (1969), Ashworth (1971), Garrod & Ashworth (1973) and Killick & Wright (1974). The life cycle starts with aggregation of amoebae, stimulated by nutritional starvation, ultimately forming a fruiting body containing spores, a cellulose sheathed stalk and a basal body. Such development of amoebae occurs synchronously in all constituent cells and at the cellular level consists of differentiation of amoebae into spore cells or stalked cells. The mechanisms by which suitable proportions of amoebae will develop into each type of cell and also become correctly located for fruiting body formation are poorly understood. This facet of the slime mould life cycle exhibits a type of pattern formation very like that which might occur in embryonic origins of higher organisms though on a much simpler scale.

One particularly interesting aspect of cellular slime mould

development is the control of aggregation of amoabae to form the multicellular "grex". Chemotaxis is now known to be induced by an agent, acrasin, which at least in part consists of 3',5'-c-AMP (Konijn et al, 1967). The concentration of c-AMP appears to be determined largely, if not entirely, by the relative activity of adenyl cyclase and a phosphodiesterase specific for c-AMP. This offers a relatively simple model of intercellular communication or "first messenger" closely akin to the hormone systems of higher organisms.

Changes in specific activity of enzymes, concentrations of enzyme substrates and products, as well as changes in RNA turnover have been considered during the life cycles of slime moulds. A considerable turnover of cellular material has been observed and it is this that allows the differentiation process to occur in the absence of exogenous materials. It is presumably the synthesis of new enzymes involved in this turnover that causes the rapid increase in synthesis of RNA. Although characteristic patterns of synthesis of certain enzymes have been recognised, it is not clear how the control of enzyme levels or activities occurs. The available evidence has prompted workers to propose quite contrasting control mechanisms. Garrod & Ashworth (1973) favour some method of genetic or transcription packaging and hence control in the amounts of enzyme synthesised, whilst Killick & Wright (1974) suggest changes in rates of degradation of enzymes to be of primary importance. Clearly there is insufficient conclusive evidence available to propose any one mechanism. Although cellular slime moulds do offer a model of differentiation that bridges the gap between the unicellular eukaryotes and higher organisms it is probably true to say that the organisms' complexity does provide something of an obstacle to an understanding of the molecular basis of differentiation.

Differentiation in the acellular slime moulds (Sauer, 1973)

is reminiscent of that in the cellular types in that nutritional and environmental triggers induce aggregation of amoebae followed by formation of a macroscopic aerial fruiting body containing spores. The important features of acellular slime moulds such as Physarum are (i) that haploid amoebae of appropriate mating types fuse to give a diploid state which lasts until the reductive division prior to sporulation; (ii) that the diploid zygotes form a large aggregate (plasmodium) with nuclear division but no cellular division; (iii) that both amoebae and these plasmodia can be induced to form resistant cysts. This complex differentiation system has been studied largely because of the interesting genetical aspects of the development and may, alongside yeast ascospore formation, result in a greater knowledge of the control of eukaryotic chromosomal mechanisms.

Having discussed some of the eukaryotic models of differentiation that have been investigated, each with its own points of interest, it must be pointed out that some of the greatest strides forward in our understanding of differentiation processes at the molecular level have been made in prokaryotes. This has, of course, largely been as a result of the greater knowledge of bacterial physiology and biochemistry. Prokaryotes have also provided a number of unusual and fascinating models, interesting in their own right but also sometimes resembling eukaryotic systems.

Differentiation at the simplest, most basic level involves the biochemistry of the cell cycle, and most important of all, the process of cell division. As a result of the unprecedented knowledge of molecular biology and genetics of E. coli it is hardly surprising that the most detailed studies of the bacterial cell cycle have been carried out on this organism (the wealth of information has been well reviewed by Donachie et al, 1973). Much work has been carried out to establish

how both initiation of DNA replication and cell division are precisely timed to occur in the cell cycle of synchronous cultures, as well as the way in which these two phenomena are coupled together. It would seem that the chromosome replication and cell division are controlled by two separable systems or "clocks". The length of the chromosome replication cycle at constant temperature remains remarkably constant for a variety of growth rates (Helmstetter & Cooper, 1968). This would indicate that there is a maximum rate at which DNA chain growth can occur at each replication fork controlling the cycle length. Control of the cell division clock is a far more complex and ill understood process although the first two-thirds of the division cycle is known to depend on protein synthesis (Pierucci & Helmstetter, 1969). The two clocks are thought to be connected in two ways. Both are thought to be initiated at about the same time, almost certainly in response to the cell doubling its "initiation mass". Also, complete cell division is dependent on both clocks and presumably therefore on the combined products of the two cycles (Jones & Donachie, 1973).

Although much is known about the biochemistry of DNA replication the in vivo process of chromosome replication, where nuclear material appears to be tightly packed together, and the segregation into two intact chromosomes presents one enormous area of unknown. Intimately connected with such problems is the determination of location and the mechanism of cell division. Models have been proposed for uni- and bidirectional growth of the cell envelope (Donachie & Begg, 1970; Autissier, Jaffe & Kepes, 1971) providing possible means by which localised regions of growth could determine sites of cell division and presumably also chromosome segregation. It has also been suggested that the site of cell division might be determined as a response to concentration gradients arising from the cell poles. Further details of some of these

problems and models will be discussed, where appropriate and relevant, later in this thesis.

Observations in E. coli have not led to conclusive mechanisms for control of periodic synthesis, just as with aspects of kinetics of enzyme synthesis in yeast cell cycles. However, it is thought that natural oscillations in repression and derepression of enzymes subject to feed-back control could give rise to the observed periodic activity, provided that some mechanism of entrainment locks in the oscillations with some other event in the cell cycle. In contrast the doublings of the inducibility of enzymes appear to be in response to gene dosage and the order of appearance of such doublings seem to closely reflect the order of structural genes on the chromosome (Donachie & Masters, 1969).

Although the molecular biology of Arthrobacter is by no means as well established as in E. coli the organism does exhibit a biochemically and morphologically well defined morphogenesis. Under certain growth conditions there is a transition from sphere to rod-shaped cells (Ensign & Wolfe, 1964). Krulwich et al,(1967a,b) were able to establish a distinct alteration in the chemical composition of cell wall mucopeptide during the transition. More recent continuous culture investigations have demonstrated that the transition results from a change in growth rate and not specifically from the presence of an environmental trigger compound (Luscombe & Gray, 1971). Ultrastructural investigations by Ward & Claus (1973) have shown that the wall of the rod-like cells, although still having a typical Gram positive structure, was considerably thinner than that of the spherical cells and concluded that this accounted for the former staining Gram negative and the latter Gram positive. Although the mechanism by which growth rate controls cell wall composition is still not known this model does offer a very simple and well defined example of the influence of cell wall composition on cell shape.

Caulobacter presents a simple yet intriguing model of differentiation (Shapiro et al, 1971). This organism has an unusual dimorphic morphology, some cells being motile "swarmer" cells and the remainder non-motile stalked cells (Stove & Stanier, 1962). The cell cycle is best considered by starting with the newly released swarmer. This cell initially has a single flagellum, many pili and a sticky holdfast at one pole. It soon loses its motility and the pili and a stalk develops at the site of the holdfast. This stalk is an envelope-bound extention of the cytoplasm and as such is classed as a "prostheca" by the definition of Staley (1968). The stalked cell then continues to enlarge and eventually develops a new flagellum, holdfast and pili at the opposite end to the stalk. Eventually binary fission occurs giving rise to the two dissimilar cells: the swarmer and the stalked cell. Selection of synchronous cultures of Caulobacter has been possible by a wide variety of methods (see introduction to Section IIB, this thesis) and consequently facilitated a number of studies of the cell division cycle. The pattern of DNA synthesis in the two post-divisional cells has been followed (Degnen & Newton, 1972a), its relationship to cell division (Degnen & Newton, 1972b), the role of transcriptional control in the cell cycle (Newton, 1972) as well as the differential absorption of phage during development (Shapiro & Agabian-Keshishian, 1970). These investigations together with a number of others have shown Caulobacter potentially to be an exceedingly valuable differentiation model. Although the body of cells enlarge in a bidirectional fashion prior to division (binary fission) rather than in a polar, unidirectional fashion (budding) and the organism is not photosynthetic it should be noted that Caulobacter does bear remarkable similarities to the budding photosynthetic bacteria to be discussed later. In particular the cell cycle, involving growth to produce two different (or differentiated) cells, forms a very

close parallel to that in Rhodopseudomonas palustris. For this reason much of the detail of Caulobacter as a model of differentiation is discussed later in this thesis.

Probably the most well established model of bacterial differentiation is that of endospore formation and outgrowth in Bacillus, which has been widely reviewed over recent years (Mandelstam, 1969; Hansen, Spiegelman & Halvorson, 1970; Mandelstam, 1971; Szulmajster, 1973; Keynan, 1973). This consideration will, for simplicity, be confined to spore formation. The sporulation of Bacillus can be regarded as consisting of a number of sequential, well defined chemical and morphological changes. This process can be induced to occur, with a fairly high degree of synchrony, by transfer of vegetative cells to a nutritionally deficient medium, or will occur when cells reach the end of exponential growth. Dawes & Mandelstam (1970) have used continuous culture studies to determine specifically that lack of glucose or nitrogen compounds are the only conditions effective in initiating sporulation. It seems quite likely that initiation of sporulation is in fact by a modified form of catabolite repression (Coote, 1974) the modification being a specific requirement for chromosome replication. Thus Mandelstam, Sterlini & Kay (1971) showed that the DNA replication which occurred on transfer of cells to sporulation medium was necessary for effective sporulation. More recently Mandelstam & Higgs (1974) have shown that there is the greatest potential of initiating sporulation about 15 min after the commencement of a round of DNA replication. This time was noted to coincide with duplication of a Stage 0 sporulation region of the chromosome and it was suggested that it might be this replication that was required, in addition to derepression of the Stage 0 operon, for initiation of sporulation. Recent evidence by Hutchinson & Hanson (1974) has indicated that a decrease in energy levels in the cells may initiate the derepression of sporulation. Perhaps this would indicate that the proposed modified catabolite

repression might in part be triggered by low energy levels.

For any study of differentiation it is necessary to determine which of the sequence of changes are essential and which are simply gratuitous. Largely as a result of studying the properties of asporogenous mutants, many of the changes that occur during the Bacillus sporulation have been classed accordingly. Chromosome mapping of such asporogenous mutants has indicated several hundred genetic loci concerned with sporulation scattered over the genome. Piggot (1973) has demonstrated that there are at least 28 operons and has suggested that several are probably activated in groups. However, the order of operons was not the same as that of their corresponding morphological stages and operons concerned with one stage were not necessarily together. Coote & Mandelstam (1973) have constructed double mutants asporogenous in two different stages. This has permitted them to determine an order of gene expression and estimate there to be something over 12 dependent sequential steps.

The control of the sequential steps in sporulation has for some time been of great interest to workers. Waites et al (1970) discussed three models for such control. Even then it was appreciated that the first model of linear sequential transcription was unlikely as gene orders did not match the order of events. It would now seem that transcriptional control may be a combination of their other two models; sequential modification of RNA polymerase and sequential induction or derepression of operons as indicated above.

Before discussing the evidence for modification of RNA polymerase it would at this point be worthwhile looking at the well established and closely comparable mechanism of control of T4 infection of E. coli (reviewed together with other systems by Travers, 1971). The core of E. coli RNA polymerase consists of four subunits: $2\alpha, \beta$ and β'. Accurate and efficient initiation of transcription also requires the

presence of another subunit, σ (sigma factor)which together with the core is described as the holoenzyme. Sequential transcription of T4 DNA, which consists of immediate-early, delayed-early and late genes depending on their times of expression, is thought to be controlled by changes of the host RNA polymerase at two levels. Although rifampicin-sensitivity is maintained, indicating no alteration of the β subunit, the evidence shows that first there is a modification of the two α subunits at about the same time as delayed-early transcription followed by a modification of the β' unit at the beginning of the late transcription. The delayed-early and late cores differ from the host core in not being stimulated to transcribe T4 DNA by low concentrations of E. coli σ. Although these changes may play a role in shutting off transcription of the host genes they would not in themselves allow phage synthesis. To permit transcription of the delayed-early T4 genes it appears that the phage codes for a new T4 sigma-like factor (Travers,1969,1970) which, with the modified polymerase core, offers a holoenzyme of new specificity.

It is now fairly well established that during the course of Bacillus sporulation there is an alteration of the vegetative RNA polymerase core enzyme (Losick & Sonenshein, 1969; Losick, Shorenstein & Sonenshein, 1970). This alteration was found to be confined to the β subunit and it is this that presumably causes rifampicin-resistant mutants either to be asporogenous or produce spores of altered morphology (Sonenshein & Losick, 1970; Doi et al, 1970). Clearly this core enzyme alteration might account for the turning off of some vegetative genes. However, the switching on of sporulation genes is another problem. Losick et al,(1970) found a slight change in the molecular weight of the σ subunit after the commencement of sporulation and suggested that it was this new σ that specified transcription of sporulation genes. On the other hand Szulmajster (1973) considers that the interaction of

vegetative σ and modified core might be responsible for initiation.

A number of other mechanisms have been proposed for transcriptional control during sporulation. Yamakawa & Doi (1971) obtained evidence for preferential transcription of DNA light strands during sporulation. Sarkar & Paulus (1972) presented evidence that suggested that peptide antibiotics of B. brevis might regulate transcription by specifically inhibiting RNA polymerase. Many observations of transcriptional control appear conflicting. Although mechanisms superficially seem well understood there is clearly much still to be determined.

Even less understood is the timing and control of commitment to sporulation. Sterlini & Mandelstam (1969) resuspended cells at different stages of sporulation in casein hydrolysate and were able to demonstrate a sequence of times of commitment (i.e. transitions from reversibility to irreversibility) to various aspects of the developmental process. The commitment time shortly preceeded a period of resistance to actinomycin D which lasted for up to 1.5 h before the particular event occurred. It was therefore concluded that the timing of commitment was controlled by synthesis of long-lived m-RNA and that the sporulation must be regulated both at transcriptional and translational levels. Although similar results have been observed in a variety of other differentiation models some workers are sceptical of the interpretations of actinomycin D studies (Killick & Wright, 1974). Indeed, a number of workers have only found evidence of normal unstable m-RNA (see Szulmajster, 1973). Further uncertainty over the problem of commitment arises concerning its timing. Several workers have observed commitment to completed sporulation at a variety of times during the development. Dawes & Mandelstam (1970) found that the nature and concentration of sporulation repressors greatly influenced the timing of apparent commitment. Perhaps

this explains a number of the contradictory results; however the question of commitment is still not satisfactoraly resolved.

Far more complex prokaryotic models have also been considered by workers, usually because of the multicellular aspects of their development and the obvious connections that these have with some eukaryotic systems. One such example is that of the life cycle of myxobacteria (Dworkin, 1972, 1973). These organisms exhibit cellular and colonial morphogenesis, in a way reminiscent of slime moulds. When their nutrient environment becomes depleted of certain amino acids aggregation of cells occurs giving rise to fruiting bodies containing differentiated resting bodies (myxospores). Studies are particularly assisted by the fact that the vegetative cell-spore cell transition can be induced without fruiting body formation in liquid culture by hydroxylated compounds such as glycerol. Additional to these features is the fact that the vegetative cells of myxobacteria move by a curious and unexplained gliding motility in a co-ordinated manner giving rise to characteristic swarms of cells. Little is known of the controls and molecular aspects of these properties. It would appear that the complexity of this prokaryote might to some extent hamper investigations, just as in the more complex eukaryotic models of differentiation. At least some of the complications in myxobacteria can be short-circuited.

A further two multicellular prokaryotic systems have been discussed as differentiation models, although they are still in their infancy when the complexity of processes within them is considered. Chater & Hopwood (1973) have reviewed the relevant aspects of differentiation in Actinomycetes. In these organisms germinated spores grow to form branched networks of hyphae within the substrate and then produce closely packed branching aerial hyphae bearing terminal chains of spores. The system has the advantage of well established genetics

but the greatest problem is that of morphogenesis in liquid culture not being possible.

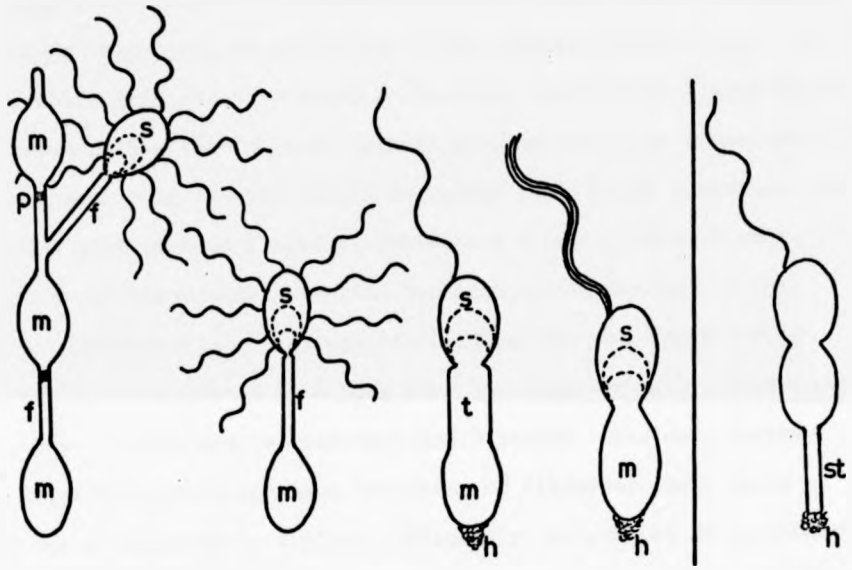
The morphological diversity and complexity of life cycles in the filamentous blue-green algae is perhaps the most extreme amongst prokaryotic organisms. However, few light photomicrographs have been published let alone ultrastructural details of the organisms. Carr & Bradley (1973) have reviewed development in the filamentous blue-green algae. The major differentiated cell types that occur are akinetes (resistant spores) and heterocysts. The latter have proved most interesting as there is evidence that they are the site of nitrogen fixation in hyphae. In addition they usually exhibit regular spacing along the length of the algal filaments. This presents an interesting model of pattern formation and intercellular control. For some time it was thought that production of ammonia, or some derivative of ammonia, as a result of nitrogen fixation by heterocysts prevented further heterocyst formation. Heterocyst formation was only considered possible a certain distance away from pre-existing ones, where the ammonia concentration was below a certain threshold. Recent continuous culture studies described by Carr & Bradley (1973) provide strong evidence that an ammonia gradient alone was not the pattern controlling factor. The authors suggested that although ammonia may prevent differentiation of a vegetative cell to form a heterocyst it might also be an early product of differentiation that would prevent development of adjacent heterocysts and therefore control pattern formation. Indeed, Wilcox, Mitchison & Smith (1973a,b) demonstrated that developing heterocysts (proheterocysts) dedifferentiated when isolated, presumably as a result of self inhibition. On the other hand isolated mature heterocysts did not regress.

Heterocysts have a number of other distinguishing features apart from the probability that they act as sites of nitrogen fixation.

They probably contain most of the organisms' poly-β-hydroxybutyrate reserves; they are the most actively reducing parts of the filaments; and, best documented of all, they lack the photosynthetic photosystem II. The significance of such features are only just being understood. As with the Actinomycetes it must be said that the morphological complexity of blue-green algae will give rise to the greatest barrier to a complete understanding of their differentiation processes.

It should be clear from this survey of existing models of differentiation that the greatest depth of understanding has been achieved when studying differentiation in unicellular organisms. The differentiation exhibited by the various models can be classified under one of two headings: obligatory or environmentally induced. Cell cycle studies, obligatory in nature, have been carried out with a variety of totally unrelated organisms and with the exception of Caulobacter demonstrating little morphogenesis. Budding and prosthecate bacteria, however, exhibit a wide range of morphogenetic features within their cell cycles which, if studied together with the biochemical changes that occur, could reveal much information about cell cycles in general and the control of morphogenesis and differentiation.

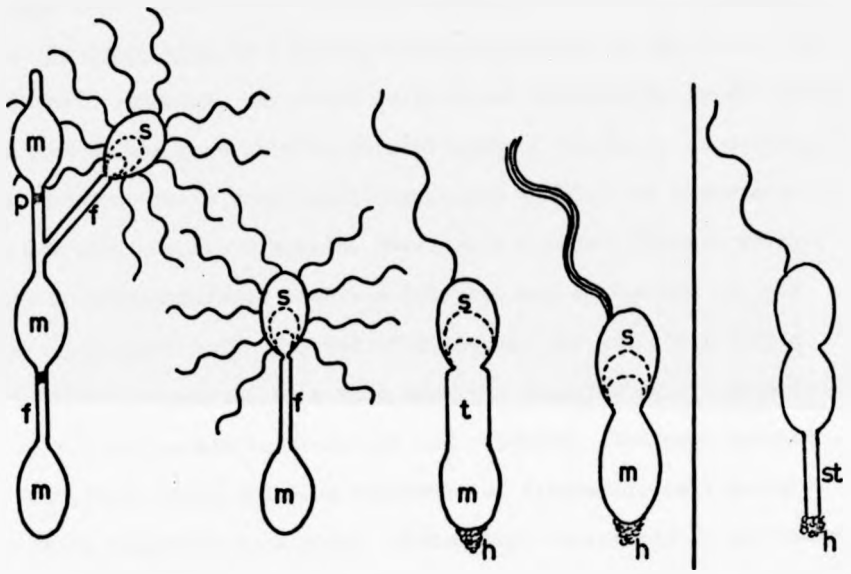
To date the only studies of the cell cycles of budding bacteria at the biochemical level that have been published are those with Hyphomicrobium (Moore & Hirsch, 1973b; Weiner & Blackman, 1973). It has become clear that the budding members of the Rhodospirillaceae (formerly Athiorhodaceae) present a group of physiologically related but morphologically differing organisms. When examined side by side it is found that they exhibit a gradient of morphogenetic complexity (Fig. 1) ideally suited to a multiple model of bacterial differentiation. Having already noted the potential of Caulobacter as a differentiation system it is interesting to note its close relationship to the cell morphology



a. b. c. d. Caulobacter

Rm. vannielii Rm. vannielii "pairs" R. palustris (& R. viridis) R. acidophila

Fig. 1. Gradient of morphogenetic complexity in budding photosynthetic bacteria. Diagrams show cells about to release swarmers and are compared with Caulobacter. All the lower cells originated by maturation of a swarmer. m, mother cell; s, swarmer (daughter) cell; f, filament; t, tube; st, stalk; p, plug; h, holdfast.



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of the budding photosynthetic bacteria (Fig. 1).

The most complex member of the group is Rhodomicrobium vannielii, first isolated by Duchow & Douglas (1949). Dow (1974) has examined Rm. vannielii as a model of differentiation in the context of a morphogenetic gradient. By slide culture and synchronous liquid culture of swarmers he has been able to unravel some of the rules concerning its mode of growth. As is very simply expressed in Fig. 1a, a swarmer cell looses its peritrichous flagella, develops a slender filament which contains no photosynthetic membrane lamellae and at the end of this produces a daughter bud. Instead of dividing, the two cells become physiologically separated by a plug near the daughter cell. This daughter cell is never motile and is therefore not released. Instead, further chain formation occurs and also branching of filaments, cell units always being separated by a plug. Ultimately, as a result of an unknown stimulus, daughter cells are produced with peritrichous flagella and these swarmers are released by constrictive division of the filament rather than plug formation occurring. A further complication to the development is the fact that under certain circumstances the terminal filaments give rise to resistant exospore bodies rather than swarmers (Gorlenko, 1969). Thus within the development of Rm. vannielii there is both obligatory and environmentally induced differentiation.

Recently, and very important to this consideration of comparative morphogenesis, it has been found that Rm. vannielii can be induced to grow by a simplified cycle of development (France & Dow, 1975). Here, the first daughter bud that forms becomes motile (Fig. 1b), is released and matures to become a mother cell capable of filament formation and further bud formation. In the meantime more swarmers are synthesised on the end of the original mother cell filament. This developmental cycle bears a very close resemblance to that observed in Hyphomicrobium (Leifson, 1964).

Not altogether different from this simplified cycle of development of Rm. vannielii are those of R. palustris and R. viridis (Whittenbury & McLee, 1967). The most striking differences are the breadth of the "tube" connecting the mother and daughter cells and the fact that they bear single sub-polar flagella and holdfast material at opposite poles (Fig. 1c). Because it is this organism, intermediate in the scale of morphogenetic complexity, that is to be the subject of this thesis its division cycle as first described by Whittenbury & McLee (1967) is simply expressed in Fig. 2. A daughter, swarmer cell (a) develops a short, blunt-ended phase contrast-translucent tube (b,c) which is slightly narrower than the original cell. At some time during this development the flagellum on the daughter cell is lost and is now known to be replaced by a sticky holdfast. At the end of the tube a bud develops (d) which eventually gives the whole structure a dumbell shape (e). Division takes place between the end of the tube and the daughter cell that has developed (f). The daughter cell, which by this time is motile, can then swim away and repeat the full cycle. The property of assymetrical division giving rise to the appendaged mother cell and the uniflagellate swarmer bears a close resemblance to Caulobacter (Fig. 1) but the essential differences are that the appendage of R. palustris has a reproductive function and that this reproduction is by budding and not binary fisson as in Caulobacter.

The most simple form of morphogenesis amongst the budding photosynthetic bacteria is exhibited by R.acidophila (Fig 1d). The cycle of development is in many respects similar to that of R. palustris with the exception that budding of the daughter cell is directly off the mother cell (Pfennig, 1969) and the swarmer is reportedly motile by a bundle of subpolar flagella (Tauschel & Hoeniger, 1974).

The budding photosynthetic bacteria not only exhibit the ill understood aspects of control in the bacterial cell cycle at varying

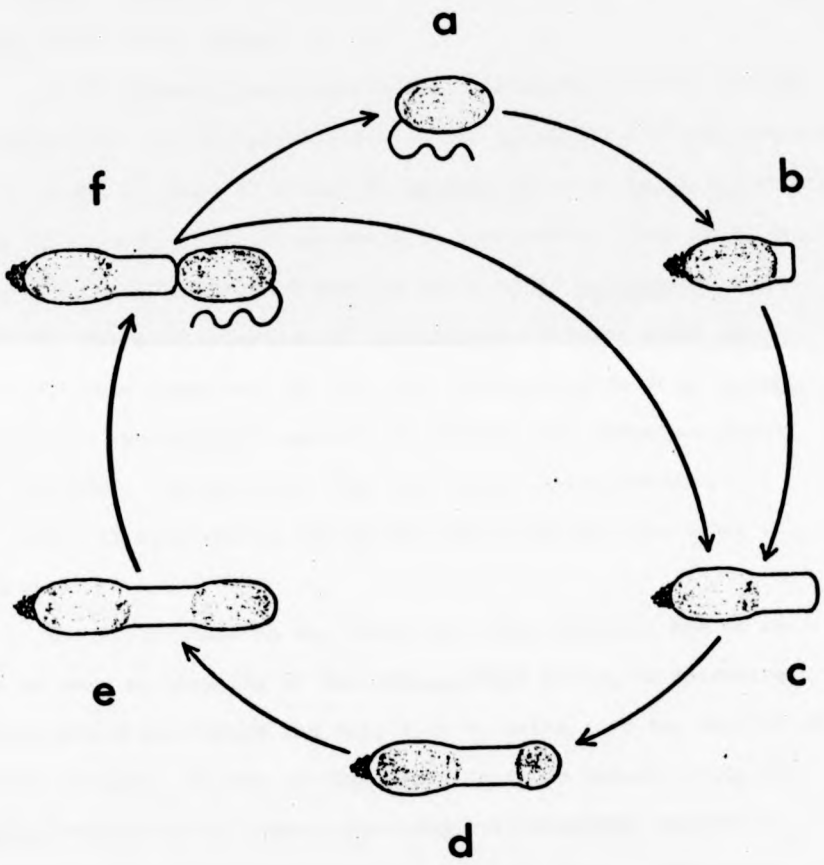

Fig. 2. Diagram of stages in the cell cycle of R. palustris. The cycle is described in the text.

degrees of complexity but also present other relevant problems not always observed in other models, such as control of cell polarity, control of cell motility cycles and the associated phototaxis, control of the induction of photosynthetic apparatus and differential control of mother and daughter cells at division.

As has already been mentioned a considerable amount of work has been carried out on differentiation in Rm. vannielii. It was therefore felt that a detailed study of either R. acidophila or R. palustris in the context of this range of organisms would be invaluable. Because of the similarity of size of mother and swarmer cells of R. acidophila it was envisaged that physical selection of synchronous cultures might be difficult with this organism. On the other hand, swarmers of R. palustris are the smallest type of cell present in cultures and therefore might easily be selected. In addition, its cell cycle is sufficiently unusual to make it interesting yet uncomplicated by multiple types of morphogenesis.

The first stage in any study of differentiation has to be to define as many as possible of the changes that occur, to determine their timing and significance and only then to delve into the control of the observed changes. It was on this basis that the present study of R. palustris was initiated, first obtaining environmental conditions resulting in consistent morphological changes with time. Under such conditions many morphological, ultrastructural and physiological changes were defined during the cell cycle and only then were attempts made to understand some of the control mechanisms of the processes.

SECTION I: EFFECT OF GROWTH CONDITIONS ON R. PALUSTRIS

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INTRODUCTION

Before embarking on a study of R. palustris as a model of bacterial differentiation it is first necessary to examine some of the effects of the environment on the appearance and rate of growth of cultures. Shapiro et al (1971) have suggested a number of criteria that should be satisfied for an organism to be suitable as a system for studying differentiation. One such criterion was that growth on defined media should be possible to permit correlation of biochemical events with morphological development. Although strains of R. palustris are known that grow in complete absence of growth factors (Klemme, 1968; Drews & Witzermann, 1971) the organism is most usually regarded as requiring p-aminobenzoic acid or some component of yeast extract (Carr, 1969). Workers have found that the presence of yeast extract stimulates the growth of most Rhodospirillaceae. Van Niel (1944) found that growth yields of Rhodospirillum rubrum were proportional not only to the concentration of acetate in the medium but also to that of yeast autolysate. In contrast, R. acidophila (Pfennig, 1969) and Rm. vannielii (Trentini, 1967) both grow at maximal rates in defined media.

Good growth of Rhodospirillaceae has been achieved using a very wide variety of organic compounds as carbon sources (Carr, 1969; Drews & Witzermann, 1971; Pfennig & Truper, 1974) although the most commonly used substrate for R. palustris is probably acetate. In his study of the growth of Rhodospirillaceae on various organic compounds, Van Niel (1944) observed considerable variation of morphology. Thus, when R. palustris was grown on malonate small apparently rod-like cells were predominant; however when crotonate or caproate were used as carbon sources, irregular branching morphology was often seen though there were

some strain differences. Changes in predominant morphological forms were even noted at different ages of batch culture; cells of widely varying lengths often appearing in star-shaped groups (rosettes) in older cultures.

Clearly then, when studying differentiation in photosynthetic bacteria it was necessary to determine in what way environmental conditions affected the morphological and biochemical properties of the cultures. Using defined conditions for growth, with perhaps the exception of the precise composition of yeast extract, it was anticipated that defined properties of heterogeneous cultures might be maintained.

During the course of any bacteriological study the facility of agar plate cultures is always a great benefit, particularly for such procedures as isolation of organisms, enumeration by viable plate counts and most techniques involving bacteriophages. Most Rhodospirillaceae can grow aerobically in the dark but do so very slowly. Aerobic plate cultures in the dark are therefore not very attractive as a culture technique. As a result, these organisms are usually cloned photoorganotrophically after serial dilution in agar deeps. However, this method does not allow colonies to be easily sub-cultured and frequently gives rise to a film of growth between the agar and the glass (Van Niel, 1944). In addition Klein & Wu (1974) have shown that enumeration of viable organisms from water by pour plates results in decreased counts when compared with spread plates, and has suggested that this is due to stresses on the organisms. Similar stresses would presumably occur when enumerating organisms by the agar deep method. Although several methods of incubating agar plates anaerobically with illumination have been described none is particularly satisfactory. In the early studies of Czurda & Maresch (1937) difficulties were encountered when plates were stacked in containers made anaerobic with pyrogallol and potassium hydroxide and incubated in the light. Their problems were probably due to the very low carbon dioxide

tension remaining as it was being absorbed by the potassium hydroxide. Skerman (1967) described a method of sealing Petri dishes onto a glass sheet together with a small amount of alkaline pyrogallol to remove oxygen. Again, lack of carbon dioxide might present problems, as well as the large amount of incubation space required being a disadvantage. Photosynthetic growth has been achieved in glass McIntosh and Fildes or Brewers jars (Swoager & Lindstrom, 1971) but these have limited capacity and illumination is non-uniform. Large polycarbonate anaerobic jars (Baltimore Biological Laboratories, BBL) give rise to a considerable amount of moisture (Collee et al, 1972) especially under illumination, which again must be non-uniform (Schmidt, Yen & Gest, 1974). The continuously gassed bag of Hill (1973) and the gassed lucite box used by Sistrom (1966) share the common disadvantage of the consumption of large amounts of oxygen-free gas, even though the latter method only had a capacity of 12 Petri dishes.

A method had, therefore, to be developed which was cheap, simple and allowed successful growth of the Rhodospirillaceae, preferably accommodating large numbers of plates and illuminated perpendicular to the surface of the plates.

MATERIALS AND METHODS

Organisms. A strain of R. palustris isolated from freshwater by Dr. C.S. Dow (University of Warwick) was used in all studies of this species, unless otherwise stated. This organism was designated strain C1. R. palustris strain 1e5 was obtained from the American Type Culture Collection (ATCC 25852). R. acidophila and Rm. vannielii were also obtained from Dr. C.S. Dow.

Media. A basal medium containing (g/l): NH_4Cl, 0.5; $MgSO_4.7H_2O$, 0.4; $CaCl_2$, 0.05 was used for cultures of R. palustris. This was usually supplemented with 1.0 g/l yeast extract and carbon sources at 1.5 g/l

unless otherwise stated, and adjusted to pH 6.9 with KOH. After autoclaving at 121° for 15 min, 50 ml/l sterile 0.1 M phosphate buffer, pH 6.9, was added to the medium.

PM medium for Rm. vannielii and R. acidophila contained (g/l): sodium pyruvate, 1.5; sodium hydrogen malate, 1.5; NH_4Cl, 0.5; NaCl, 0.4; $MgSO_4.7H_2O$, 0.4; $CaCl_2$, 0.05. The pH was adjusted to 6.9 (Rm. vannielii) or 5.8 (R. acidophila) with KOH before autoclaving at 121° for 15 min. After sterilisation, 50 ml of sterile 0.1 M phosphate buffer of the appropriate pH were added to each litre of medium.

Where solidified media were required, 15 g/l Bacto-agar (Difco Laboratories, West Molesey, Surrey) were added to the liquid media before sterilisation.

Phosphate buffers were prepared from mixtures of 0.1 M $Na_2HPO_4.12H_2O$ (35.85 g/l) and 0.1 M $NaH_2PO_4.2H_2O$ (15.6 g/l) in proportions which gave the desired pH.

Cultivation of organisms. Organisms were usually maintained in photosynthetic sub-cultures. For this purpose 250 ml conical flasks fitted with B19 ground glass necks and stoppered with No. 37 standard turn-over type Suba-Seals (William Freeman & Co. Ltd., Staincross, Barnsley, Yorks.) were most frequently used as culture vessels, although smaller stoppered bottles were also employed. Cultures were gassed with oxygen-free nitrogen for about 10 min via syringe needles through the Suba-Seals. Incubation of flasks was either on a warm room rotary shaker or in a reciprocating water bath with illumination provided by tungsten bulbs in desk lamps.

Semi-aerobic cultures were grown by incubating 50 ml cultures in cotton wool-plugged, foil-wrapped 250 ml conical flasks, without any agitation. Aerobic cultures were prepared in a similar fashion but agitated vigorously on a rotary shaker.

Cuvette cultures. Small scale liquid cultures for optical density measurements were prepared by filling to the brim and tightly stoppering 1 cm round-topped glass cuvettes. Between readings cuvettes were incubated horizontally in a water bath and illuminated from above.

Spectrophotometry. All bacterial spectra and optical density measurements were made using 1 cm glass cuvettes in a Pye-Unicam SP 500 spectrophotometer.

Light microscopy. Light microscopy was carried out either on a Leitz Orthoplan microscope (E. Leitz (Instruments) Ltd, Luton, Beds.) fitted with an Orthomat camera and Heine phase contrast condenser or on an Olympus EHT microscope (Olympus Optical Co. Ltd, Tokyo, Japan) with a PM-6 camera and phase contrast optics. Photomicrographs were taken on Panatomic-X film (Kodak Ltd, Hemel Hempstead, Herts.) developed with Contrast FF developer (Ilford Ltd, Ilford, Essex) and printed on Ilfobrom (Ilford) grade 5 paper.

Electron microscopy. Washed cells were dried onto Formvar coated electron microscope grids. The preparations were either shadowed with gold-palladium alloy in an AEI vacuum coating unit or negative-stained with 0.5% uranyl acetate and then examined using an AEI Corinth 275 electron microscope. Electronmicrographs were taken on Ilford 70 mm line film, N4E50.

Gas chromatography. Oxygen and nitrogen were separated and measured by gas chromatography of 0.5 ml samples in a Pye-Unicam Series 104 chromatograph with a katherometer head. Chromatography was through a seven foot column of 60-80 mesh Molecular Sieve 5A (Pye-Unicam Ltd, Cambridge) at 50°, with helium carrier gas at 30 ml/min and a bridge current of 240 mA. Peak areas on the output trace produced by a linear recorder were calculated using an associated mechanical integrator. Oxygen content of the samples was calculated as its percentage of the total oxygen + nitrogen peak areas.

Flagella stain. Flagella were stained by the method of Rhodes (1958).

Preparation of anaerobic bag(Fig. 3). Inoculated agar plates were stacked up to three deep, lids uppermost, and taped together. The addition of an empty dish on the top of the stack minimised condensation in the top plate. Up to six stacks were placed on a 27 x 30 cm plastic tray which was covered with aluminium foil. This tray and a 100 ml beaker containing 20 ml saturated pyrogallol were then placed inside a 30 x 46 cm Nylon bag of 0.05 mm thickness (Portex Ltd, Hythe, Kent). A layer of foil was placed above and below the open end of the bag which was then sealed with a domestic soldering iron (Fig. 4). At each end of the seal necks were formed into which were inserted 6 mm glass tubes which were held in place by tightly binding with adhesive tape. After gassing the bag through rubber vent tubes with oxygen-free nitrogen for about 30 to 60 min, 20 ml of alkali (10% NaOH + 15% K_2CO_3) were injected by syringe through the top surface of the bag into the pyrogallol. The needle hole was sealed with adhesive tape. A second gassing lasting 30 min was usually necessary a few hours later to flush out oxygen that had originally been trapped in the Petri dishes.

After use, anaerobic bags were opened by cutting off the end opposite to the vents. Bags with the ready made gassing vents could then be re-used 4 to 5 times, only having to make a straight seal prior to gassing and incubation.

RESULTS

Nutrient media and growth conditions

The growth rate of R. palustris in basal medium plus 1.5 g/l sodium hydrogen malate and 1.0 g/l yeast extract (MYE; as previously used at University of Warwick) was rather slow. Indeed, if the yeast extract

Fig. 3. Sealed bag for anaerobic illuminated incubation of agar plates.

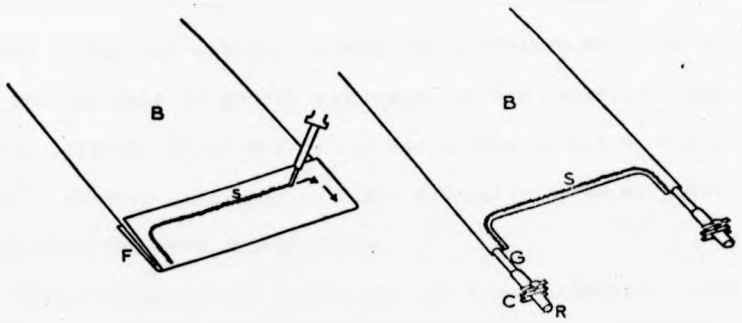

Fig. 4. Seal and vents of anaerobic bag. B, Nylon bag; S, line of seal; F, aluminium foil; G, glass vent tube; R, rubber tube; C, tube clip.

was omitted substantial increase in the turbidity of cultures was only seen after 4 to 5 d.

In order to rapidly observe the effect of changing various parameters in the growth media the rate of optical density increase in cuvette cultures of R. palustris was examined. Extinction spectra of R. palustris in nutrient medium (Fig. 5) indicated that only bacteriochlorophyll a absorption peaks at about 810 nm and 865 nm were detectable above the general light scattering (optical density) spectrum. Consequently, growth of R. palustris was followed at 540 nm (optical density) and 810 nm (optical density plus bacteriochlorophyll a). Experiments were carried out in groups of three (Table 1) and it is only within these groups that rates of optical density increase could strictly be compared. The optical density doubling time of R. palustris in MYE, incubated at 30° with 1,500 lux illumination was found to be about 10 h. Supplementing MYE with 1.0 g/l casamino acid had little effect on the growth rate. However, when the sodium hydrogen malate concentration in MYE was increased to 2.5 g/l the doubling time was retarded to more than 12 h. Growth on MYE at 1,500 lux using various temperatures of incubation indicated that 30°was sub-optimal, growth being fastest at 36 to 40°. Only at 44° was the rate of growth suppressed by the increased temperature of incubation. Altered pH of MYE medium had little effect on the rate of growth at 40°. However, increase in light intensity up to at least 8,750 lux did give improved growth rates.

Using the new found optima of high light intensity (8,000 lux was used) and higher temperature (36 to 40°), the influence of the type of carbon source on growth rate was examined. Sodium acetate, sodium pyruvate or sodium glutamate was used as a carbon source together with basal medium plus yeast extract. Glutamate was unsuitable as a carbon source but pyruvate and acetate both gave doubling times of less than 5.5 h. When

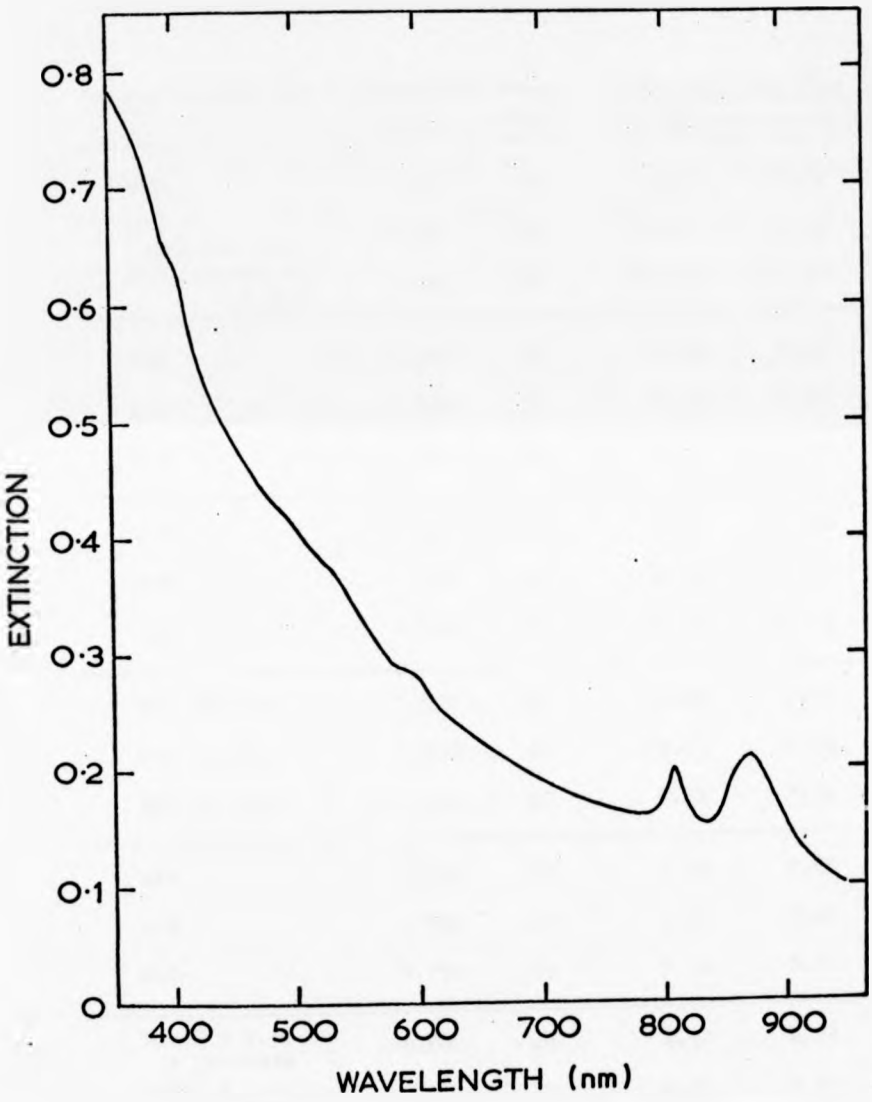

Fig. 5. Whole cell absorption spectrum of R. palustris in nutrient medium.

Table 1. Effect of growth medium, temperature and light intensity on rate of growth of R. palustris. Increases in extinction at 540 nm and 810 nm were followed in cuvette cultures.

medium composition	illumination (lux)	temp. (°C)	O.D. doubling time (h) at 540 nm	at 810 nm
MYE	1,500	30	9.97	10.74
MYE + casamino acid	1,500	30	11.07	11.07
MYE (malate at 2.5 g/l)	1,500	30	>12.00	>12.00
MYE	1,500	32	8.14	7.87
MYE	1,500	36	6.20	6.27
MYE	1,500	40	5.74	5.67
MYE	1,500	38	6.34	5.94
MYE	1,500	42	6.74	6.67
MYE	1,500	44	>12.00	11.74
MYE, pH 6.8	1,500	40	8.47	8.20
MYE, pH 6.4	1,500	40	8.07	7.74
MYE, pH 6.0	1,500	40	8.34	7.94
MYE	1,500	40	9.54	9.87
MYE	3,500	40	9.47	9.20
MYE	8,750	40	5.74	5.67
basal + y.e. + pyruvate	8,000	40	4.87	4.74
basal + y.e. + acetate	8,000	40	4.47	4.27
basal + y.e. + glutamate	8,000	40	>12.00	>12.00
basal + y.e. + pyr + ac	8,000	40	3.54	3.40
basal + y.e. + mal + ac	8,000	40	4.34	4.20
basal + y.e. + mal + pyr	8,000	40	4.14	3.67

Table 1. Effect of growth medium, temperature and light intensity on rate of growth of R. palustris. Increases in extinction at 540 nm and 810 nm were followed in cuvette cultures.

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MYE (malate at 2.5 g/l)	1,500	30	>12.00	>12.00
MYE	1,500	32	8.14	7.87
MYE	1,500	36	6.20	6.27
MYE	1,500	40	5.74	5.67
MYE	1,500	38	6.34	5.94
MYE	1,500	42	6.74	6.67
MYE	1,500	44	>12.00	11.74
MYE, pH 6.8	1,500	40	8.47	8.20
MYE, pH 6.4	1,500	40	8.07	7.74
MYE, pH 6.0	1,500	40	8.34	7.94
MYE	1,500	40	9.54	9.87
MYE	3,500	40	9.47	9.20
MYE	8,750	40	5.74	5.67
basal + y.e. + pyruvate	8,000	40	4.87	4.74
basal + y.e. + acetate	8,000	40	4.47	4.27
basal + y.e. + glutamate	8,000	40	>12.00	>12.00
basal + y.e. + pyr + ac	8,000	40	3.54	3.40
basal + y.e. + mal + ac	8,000	40	4.34	4.20
basal + y.e. + mal + pyr	8,000	40	4.14	3.67

combined pairs of malate, acetate and pyruvate were compared as carbon sources at 40° and 8,000 lux illumination, the best doubling time of about 3.5 h was obtained using 1.5 g/l sodium acetate and 1.5 g/l sodium pyruvate.

The medium used in further studies on R. palustris contained basal medium plus 1.5 g/l sodium pyruvate, 1.5 g/l sodium acetate and 1.0 g/l yeast extract (PAYE medium). Since considerable heat was produced by illumination at 8,000 lux, this was reduced to about 4,000 lux as a compromise. Satisfactory growth rates could be obtained at 36° but 34° could be used if a slower growth rate was desirable. Where warm room facilities were necessary during experiments, incubation had to be at 30°.

Growth in defined medium

Growth of R. palustris C1 is possible in completely defined media by omitting yeast extract, however, the presence of yeast extract greatly stimulates growth. In order to establish what factors in yeast extract were stimulatory a culture was grown in yeast extract-free basal medium plus pyruvate and acetate (PA) and 20 ml aliquots of PA, supplemented with combinations of vitamins and amino acids (Table 2), were each inoculated with 0.025 ml of culture. Each culture was gassed with oxygen-free nitrogen for 1 min and all were incubated at the same light intensity at 30°. Comparative growth determined visually after 3 and 6 d (Table 3) indicated that growth stimulation was due to vitamins and in particular p-aminobenzoic acid (PABA), biotin, folic acid and calcium pantothenate. A comparison of growth in PA supplemented with combinations of these four vitamins (Table 4) revealed greatest stimulation by calcium pantothenate with PABA or folic acid, but not using PABA with folic acid. Biotin did not seem to be stimulatory. Since PABA is a precursor of folic acid it would seem that the organism is inefficient at synthesising the PABA moiety of folic acid and is also able to grow faster when pantothenate is provided, presumably as a precursor of

Table 2. Amino acid and vitamin pool matrix for investigating growth stimulation of R. palustris in PA medium.

pool No.	1	2	3	4	5	6
7	L-leu	L-OH pro	DL-val	DL-nor leu	biotin*	PABA*
8	gly	L-arg	DL-met	DL-βphe	folic acid*	cyanocobalamin*
9	DL-ileu	DL-orn	L-tyr	DL-3,4OHphe	pantothenate*	choline Cl*
10	L-his	DL-asp	L-cys HCl	DL-ser	pyridoxine*	inositol*
11	L-lys	L-pro	L-cys	DL-try	ascorbic acid*	riboflavin°
12	DL-thr	DL-ala	L-glu	DL-2 amino-butyrate	thiamine HCl×	nicotinic acid*
13	biotin*	folic acid*	pantothenate*	PABA*		
14	pyridoxine*	ascorbate*	thiamine*	PABA*		
15	+ yeast extract (1.0 g/l)					
16	- yeast extract					

Pools 1 to 12 as described by Holliday (1956).

*, 50 µg/l final concentration

o, 25 µg/ml final concentration

x, 5 µg/ml final concentration

all others, 5 mg/l final concentration

Table 3. Estimated growth of R. palustris on PA supplemented with amino acid and vitamin pools (as in Table 2).

	estimated growth							
pool No.	1	2	3	4	5	6	7	8
3 days	+	+	+	+	++	++	+	+
6 days	+	+	+	+	+++	+++	+++	++
	estimated growth							
pool No.	9	10	11	12	13	14	15	16
3 days	+	+	+	+	+++	++	+++++	+
6 days	+	+	+	+	+++	+++	+++++	+

+ to +++++, arbitrary increasing scale of growth

Table 4. Estimated growth of R. palustris on PA supplemented with combinations of vitamins.

bottle No.	supplement (5 mg/l)				growth
	PABA	biotin	folic acid	pantothenate	2d
1	x	x	x	x	+++
2	x	x	x		+
3	x	x			+
4	x		x		+
5	x			x	++
6		x	x		+
7		x		x	+
8			x	x	++
9	all vitamins (pools 5+6 of Table 2)				+++
10	+ yeast extract (1.0 g/l)				+++++
11	- yeast extract				+

+ to +++++, arbitrary increasing scale of growth

coenzyme A. It is important to note that the apparent growth with any of the vitamin supplements was never as much as with yeast extract.

Comparison of aerobic with anaerobic growth

Although R.palustris was normally cultured photosynthetically, aerobic growth could also be achieved. Normal budding morphology, closely resembling the organism when grown photosynthetically (Fig. 6), was observed when R. palustris was grown for several sub-cultures semi-aerobically. These cultures were slightly pink indicating a low level of photopigment synthesis. However, when the organism was sub-cultured in vigorously aerated flasks normal morphology was only seen for the first two sub-cultures. Visible growth was obtained within 2 d incubation of each of at least eight subsequent sub-cultures, but branching, "knobbly", elongated forms of cells were observed (Fig. 7). The cultures showed no sign of pigmentation. The swellings on the cells were almost invariably nearer one end suggesting that they were concerned with some interference of the normal assymetrical division process in R. palustris. It is interesting to note that a similar swelling appearance at the division site of R. acidophila was also observed during unfavourable conditions of growth (Fig. 8).

In order to compare the whole cell absorption spectrum of R. palustris when grown aerobically and anaerobically cells were suspended in saturated sucrose solution. This enabled cells to remain suspended indefinitely and also reduced light scattering (turbidity) to a minimum. The absorption spectrum for aerobically incubated, PAYE-grown cells (Fig. 9) revealed complete absence of absorption peaks. In contrast, anaerobic growth in the light gave rise to a spectrum with a carotenoid peak at 498 nm (with shoulders at 465 nm and 532 nm) and bacteriochlorophyll a peaks at 375, 591 807 and 865 nm (Fig. 9).

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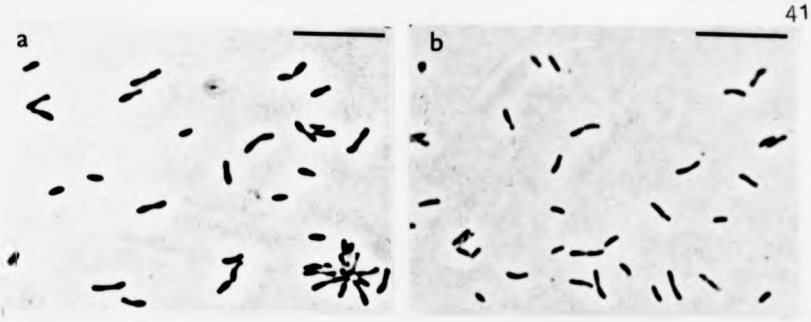

Fig. 6. Photosynthetic (a) and semi-aerobic (b) growth of R. palustris on PAYE medium. Bar represents 10 µm.

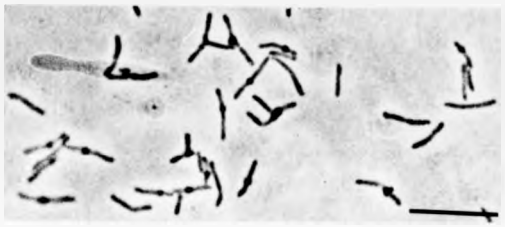

Fig. 7. Abberent morphological forms of R. palustris grown in a vigorously aerated culture. Bar represents 10 µm.

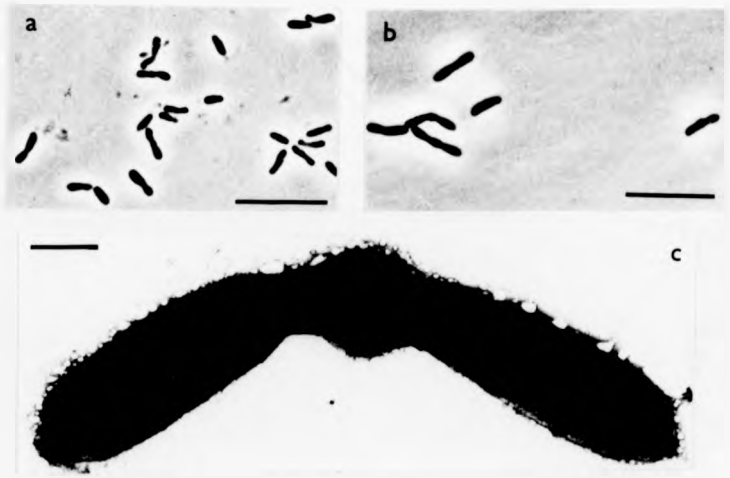

Fig. 8. Normal appearance of R. acidophila (a) compared with abnormal cells having swollen division planes (b & c). a & b, bar represents 10 µm; electronmicrograph of negative-stained preparation, c, bar represents 0.5 µm.

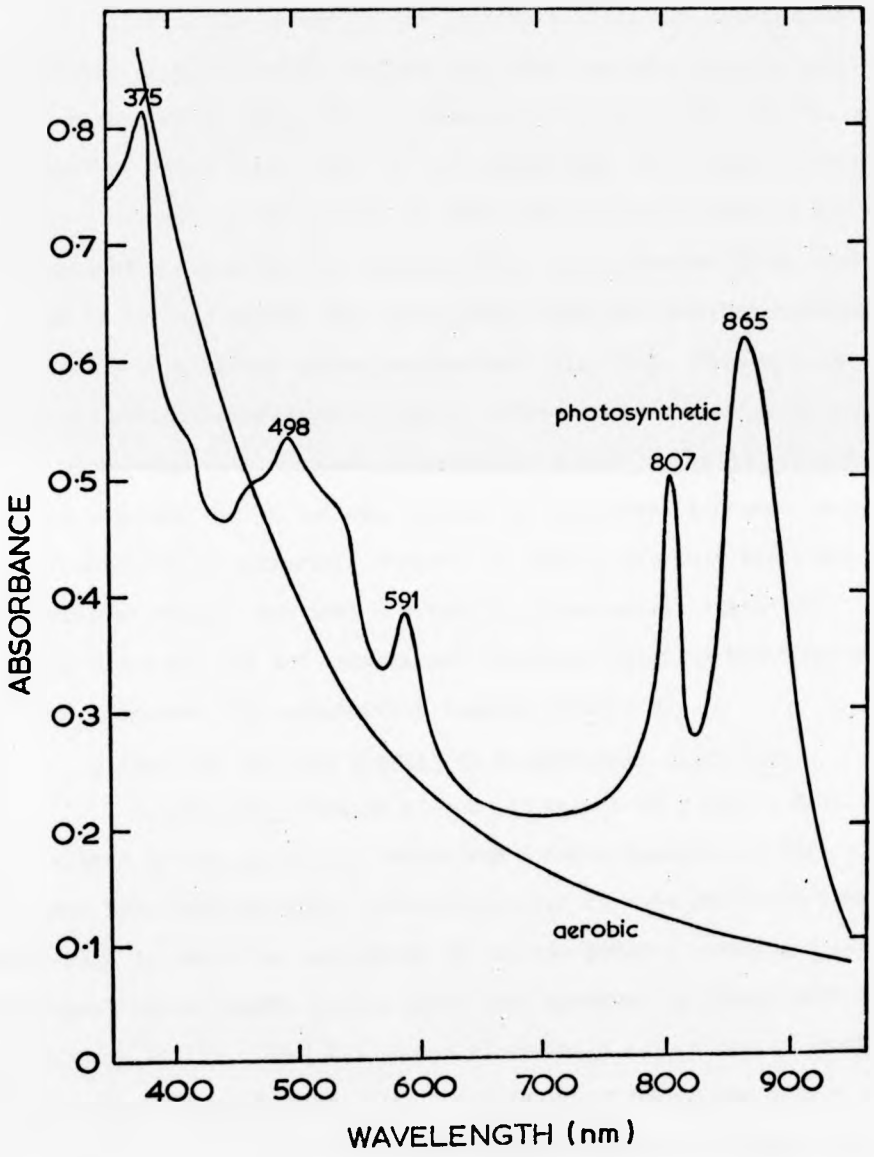

Fig. 9. Whole cell spectra of R. palustris grown photosynthetically and aerobically. Cells were resuspended in saturated sucrose solution.

Effect of culture conditions on motility and rosette formation

During the course of the initial aerobic and photosynthetic sub-cultures of R. palustris in PAYE the organisms were seen to produce motile swarmer cells (Fig. 10) and large polarly aggregated clumps, or "rosettes" of cells (Fig. 11a). It was noted that after some weeks these two properties were largely lost, daughter cells mostly becoming non-motile and clumps of a few cells only occasionally being observed (Fig. 11b). Electron microscopy showed that cells still produced holdfast material but flagella were either absent or abnormal (Fig. 12). Temperature, light intensity, medium concentration, carbon source, presence of yeast extract, amount of gassing with nitrogen, addition of oxygen or carbon dioxide, inoculum size and age of culture all had no consistent influence on the lack of motility of cultures. However, it did appear that there was an environmental trigger and that a mutation to non-motility had not occurred since on rare and unexplained occasions motility was temporarily regained, together with substantial rosette formation.

Stimulation of tube elongation by phosphate starvation

R. palustris strains C1 and 1e5 were both grown in PAYE, the cells washed in phosphate-free medium and a heavy inoculum of these cells incubated anaerobically under illumination for 24 h in phosphate-free PAYE. Fig. 13 shows the comparison of the two strains before and after phosphate-limited growth. Short tubes were apparent in normal growth of R. palustris C1 (Fig. 13a) but became elongated 4 to 5 times on growth without added phosphate (Fig. 13b). Strain 1e5 normally has only a short tapering tube (Fig. 13c) but phosphate starvation also gave rise to a 4 to 5-fold elongation of the tube in this strain (Fig. 13d). Close examination of photomicrographs indicated that phase contrast-opaque areas of the cells, which indicate the location of photosynthetic membrane lamellae, extended into the terminal regions of the elongated

Fig. 10. Normal flagella of R. palustris. a, light photomicrographs of cells stained by Rhodes' method (Rhodes, 1958), bar represents 10 μm; b, shadowed electronmicrograph, bar represents 0.5 μm.

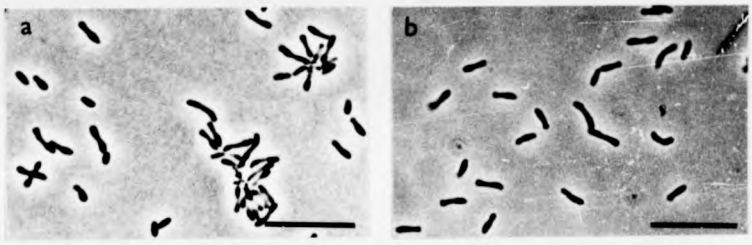

Fig. 11. Appearance of motile (a) and non-motile (b) cultures of R. palustris. Bar represents 10 μm.

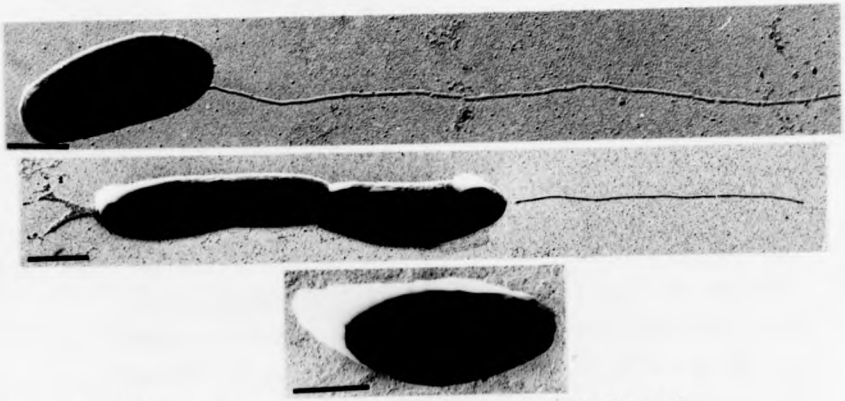

Fig. 12. Shadowed electronmicrgraphs of flagellate and non-flagellate non-motile R. palustris cells. Bar represents 0.5 μm.

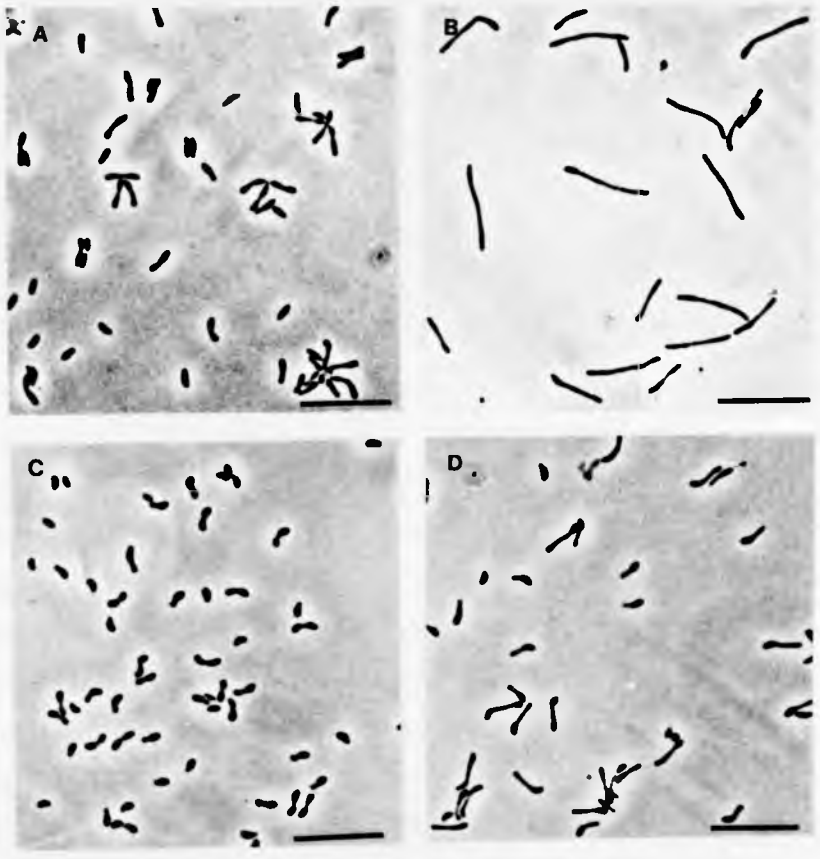

Fig. 13. The effect of phosphate-limited growth on morphology of R. palustris. Normal growth of strain C1 (a) was compared with phosphate-starved growth (b); and normal growth of R. palustris strain 1e5 (c) with its growth under phosphate starvation (d). Bars represent 10 μm.

tubes resulting from phosphate starvation. Under normal growth conditions these phase contrast-opaque areas were completely absent from the tubes.

Growth of organisms on agar plates

In order to grow Rhodospirillaceae anaerobically on agar plates it was necessary to develop a method of illuminated, anaerobic incubation of Petri dishes. The anaerobic bag system (Fig. 3) and its preparation is described in the Materials and Methods section. The anaerobiosis of the system was tested by gas chromatography of samples taken from a bag during the course of 9 d incubation with 1,500 lux illumination in a room at 30°. During this period oxygen levels remained at an estimated level of 0.104% ± S.D. 0.016%. An example of the oxygen and nitrogen separation by gas chromatography of air in anaerobic bag samples is shown in Fig. 14. A sample taken by syringe from a cylinder of "oxygen-free" nitrogen showed the presence of 0.045% oxygen; however, when the cylinder was sampled directly by a closed system only 0.017% oxygen could be detected. Since sampling with a syringe would seem to cause slight contamination with air, oxygen content of the gas samples from the Nylon bag was presumably lower than the levels detected. Further evidence that oxygen was at a very low level was provided by the pyrogallol which was only beginning to brown appreciably after 6 to 7 d. Even though it was absorbing oxygen, as shown by the development of the brown colour, the pyrogallol was capable of maintaining an oxygen level low enough to allow growth of R. acidophila, R. palustris and Rm. vannielii, even when unsatisfactory seals had permitted exchange of oxygen between the atmosphere and the bag. The fact that Nylon film is permeable to water vapour meant that there was little accumulation of moisture in the bag even under illumination.

Good photosynthetic growth of the three Rhodospirillaceae tested was achieved by this method in times considerably less than those required for aerobic growth (Fig. 15). All photosynthetic colonies were

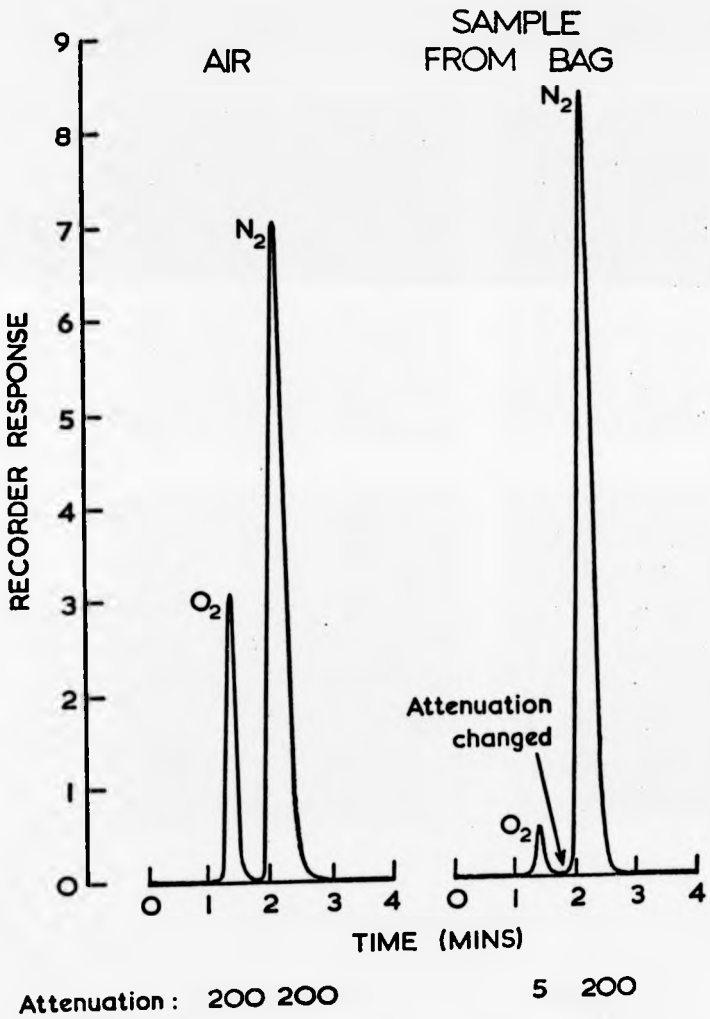

Fig. 14. Separation of oxygen and nitrogen by gas chromatography. The air sample gave an oxygen peak area indicating 21.0% oxygen. The sample from an anaerobic bag gave an oxygen peak corresponding to 0.101%. [Note that the attenuation used for the latter gave a 40-fold increase in sensitivity]

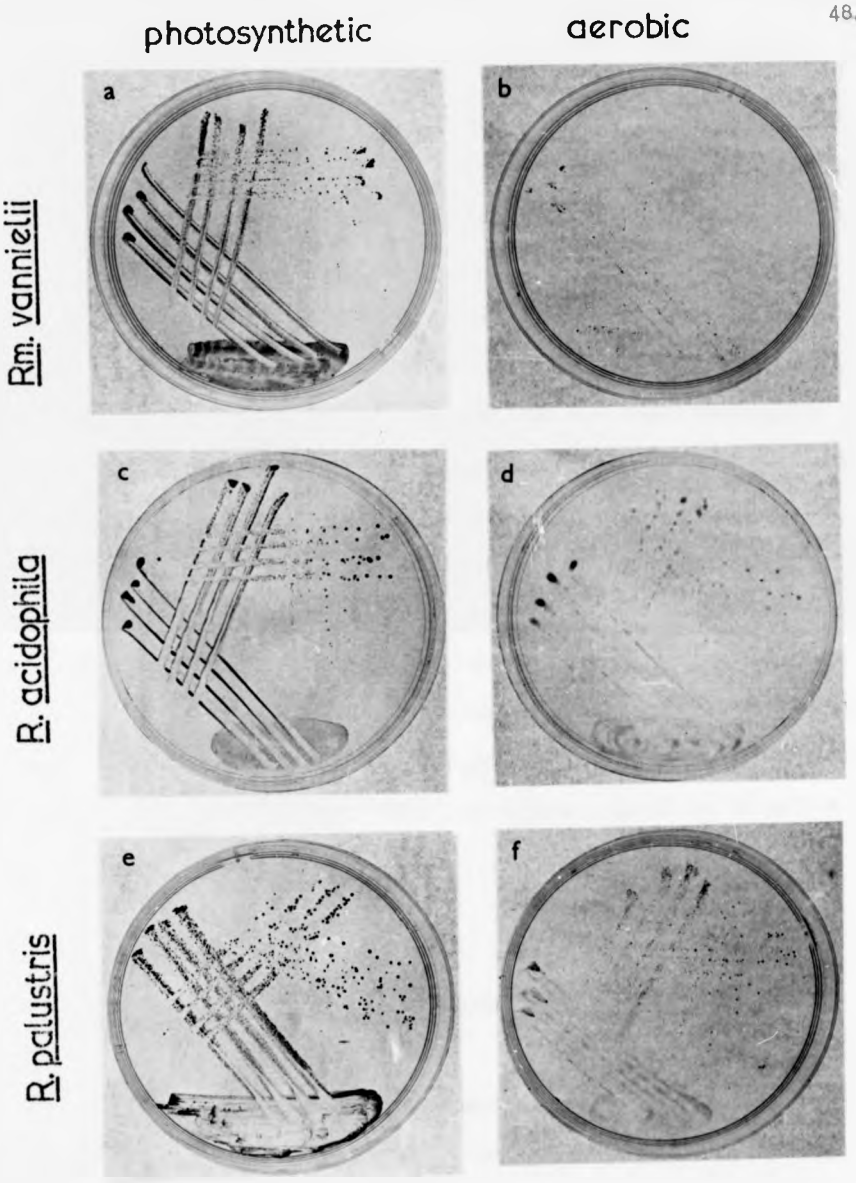

Fig. 15. Photosynthetic and aerobic cultures of some Rhodospirillaceae. 5 d photosynthetic (a) and 18 d aerobic (b) culture of Rm. vannielii grown on pH 6.8 PM medium; 5 d photosynthetic(c) and 12 d aerobic (d) culture of R. acidophila grown on pH 5.8 PM medium; 4 d photosynthetic (e) and 12 d aerobic (f) culture of R. palustris grown on PAYE medium.

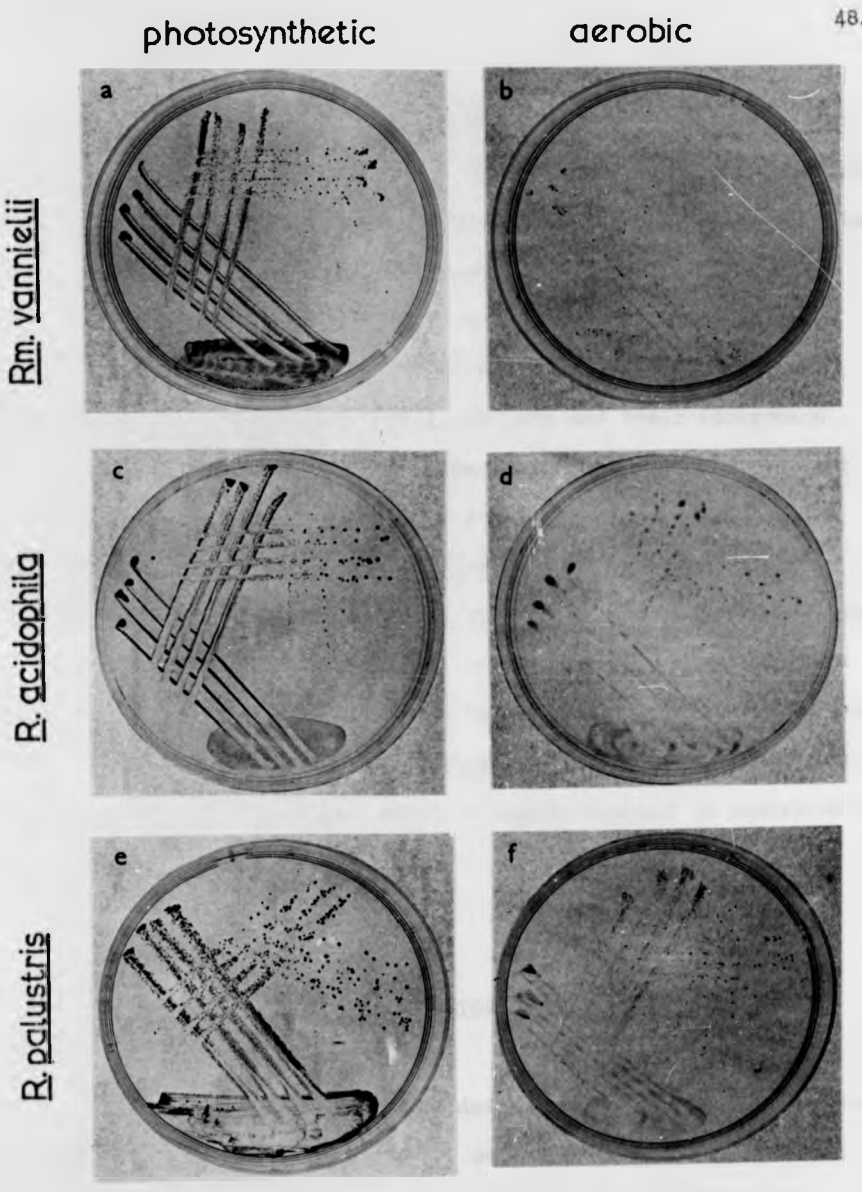

Fig. 15. Photosynthetic and aerobic cultures of some Rhodospirillaceae. 5 d photosynthetic (a) and 18 d aerobic (b) culture of Rm. vannielii grown on pH 6.8 PM medium; 5 d photosynthetic(c) and 12 d aerobic (d) culture of R. acidophila grown on pH 5.8 PM medium; 4 d photosynthetic (e) and 12 d aerobic (f) culture of R. palustris grown on PAYE medium.

pigmented uniformly, whereas aerobic cultures grown in the dark formed white colonies with pigmented centres. This was presumably due to pigment synthesis being induced by low oxygen tensions at the centre of colonies.

Agar plates streaked with samples from Winogradsky bottle enrichments allowed direct isolation of a number of photosynthetic organisms. Amongst these were a few isolates resembling R. palustris. Several isolates produced greeny-brown cultures and their absorption spectra in sucrose (Fig. 16) revealed the presence of bacteriochlorophyll b with absorption maxima at 400, 605, 820 and 1015 nm and what were presumed to be carotenoids with absorption maxima at 420, 455 and 485 nm. The pigment content, motile appearance, rosette formation and morphology (Fig. 17) indicated that these isolates were R. viridis. Two isolates produced deep reddish-brown colonies and had actively motile spirillum-type cells (Fig. 18), typical of Rhodospirillum sp. It would seem, therefore, that the anaerobic bag technique could be widely applied to isolation and culture of Rhodospirillaceae.

DISCUSSION

In attempting to determine defined growth conditions to give short doubling times with a predictable cell morphology, the most significant effects on growth rate were obtained by incubating at 36 to 40° in high light intensities using media containing yeast extract and carbon sources such as malate, acetate and pyruvate.

Although several workers have grown R. palustris at 30° (Whittenbury & McLee, 1967; Chernyad'ev, Kondrat'eva & Doman, 1970; Drews & Witzermann, 1971) the higher temperature optima of many strains have been recognised for some time (Van Niel, 1944). R. palustris strain C1

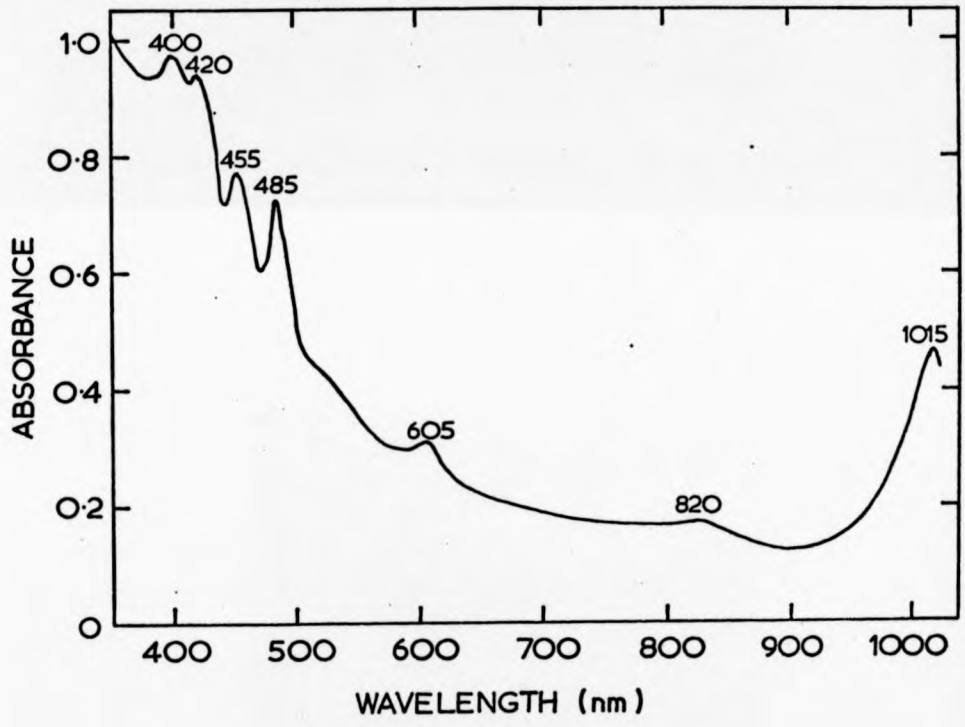

Fig. 16. Whole cell spectrum of presumptive R. viridis isolate. Cells were suspended in saturated sucrose solution.

Fig. 17. Morphology of presumptive R. viridis isolate. Bar represents 10 μm.

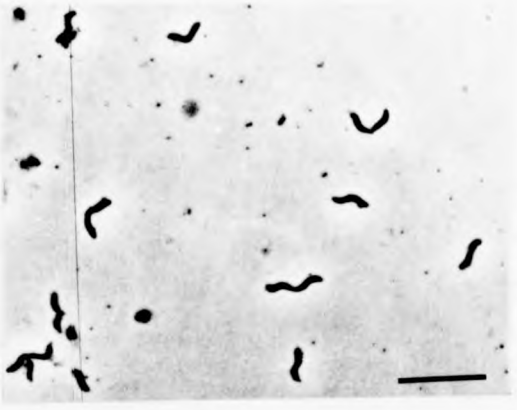

Fig. 18. Morphology of presumptive Rhodospirillum isolate. Bar represents 10 μm.

was isolated from freshwater in Scotland (Dow, 1974) and would therefore not be expected to have the high temperature optimum that it exhibits. It is interesting to note that purple photosynthetic bacteria have been found in sulphur hot springs at temperatures up to 90° (Kondrat'eva, 1965). Gaffron has observed a five-fold increase in the rate of CO_2 assimilation by R. palustris when the temperature was raised from 25° to 40° (cited by Kondrat'eva, 1965) and it is therefore probable that metabolism in strain C1 may be more efficient at the higher temperatures.

Increased illumination of cultures of Rhodospirillaceae is almost invariably found to increase the rate of growth, although above certain limits of illumination the growth rate increase becomes suppressed. In R. spheriodes this light saturation has been observed at about 10,000 lux (Sistrom, 1962), whilst in Rhodospirillum rubrum it was somewhat lower at about 4,000 lux (Holt & Marr, 1965). In both of these organisms it was found that the specific bacteriochlorophyll content responded to light intensity as a reciprocal of the growth rate. Holt & Marr (1965) were able to show that regulation of pigment was achieved by varying membrane content of the cells whilst having constant concentration of bacteriochlorophyll in the membranes. Only at very high light intensities did the pigment concentration in membranes decrease. In R. palustris light saturation did not seem to occur up to 8,750 lux, but it could be anticipated that cells grown at this light intensity would have a low content of photosynthetic membranes. In studying photosynthetic membrane formation during the cell cycle a high membrane content of cells would certainly be an advantage but this would be accompanied by a low growth rate. In addition to difficulties with heating by the illumination source, this is another reason why a compromise light intensity was used.

Workers have described growth of R. palustris on a variety of carbon compounds ranging from bicarbonate, pyruvate, lactate, malate,

succinate, ethanol and glycerol through to formate, acetate and higher fatty acids (Van Niel, 1944; Pfennig, 1969; Drews & Witzermann, 1971; Pfennig & Truper, 1974). Although good growth of R. palustris was achieved using sodium hydrogen malate and modified environmental conditions, acetate and pyruvate were also found to be very good carbon sources, in agreement with other experimentors observations. Lascelles (1959) and Keane et al (1963, cited by Carr, 1969) obtained good growth of R. palustris on media containing glutamate although other carbon sources such as malate were present. In agreement with the observations of Drews & Witzermann (1971) the strain of R. palustris used in this work only gave poor growth on glutamate. Growth on the combined carbon sources of acetate and pyruvate was marginally the best. Van Niel (1944) demonstrated that purple bacteria can convert acetate carbon into cell material with a very high efficiency. Chernyad'ev, Kondrat'eva & Doman (1970) showed that acetate is assimilated in R. palustris via the tricarboxylic acid cycle and glyoxylate cycle both in light and darkness. Pyruvate is most likely to be converted into acetyl-CoA, initially by the action of pyruvate decarboxylase (Krassilnikova & Kondrat'eva, 1974) and then assimilated in the same way as acetate. It is conceivable that pyruvate might be converted into phosphoenolpyruvate (Buchanan & Evans, 1966). This intermediate could act as a precursor of 3-phosphoglycerate for carbohydrate metabolism, as in Rhodospirillum rubrum (Evans, 1965), or be carboxylated to oxaloacetate (Chernyad'ev, Uspenskaya & Doman, 1972) and then incorporated into the tricarboxylic acid cycle.

It is perhaps significant that growth of R. palustris strain C1 was stimulated in defined medium by pantothenate as well as PABA. Since pantothenate is a part of coenzyme-A adequate levels of this cofactor would be essential for primary steps in the assimilation of both acetate and pyruvate. It is important to stress that some unknown factor(s) is

present in yeast extract which causes considerable stimulation of growth of most strains of R. palustris. Similar observations have been made with R. viridis (Drews & Giesbrecht, 1966) although R. acidophila (Pfennig, 1969) and Rh. vannielii (Duchow & Douglas, 1949; Trentini, 1967) are not stimulated or are even inhibited by yeast extract.

Aerobic growth of R. palustris is purely by respiratory metabolism using the tricarboxylic acid cycle and oxidative phosphorylation via cytochromes with oxygen as the terminal electron acceptor (King & Drews, 1975). It is the oxygen tension which inhibits pigment synthesis even in light (Tauschel & Drews, 1967) and a number of workers have found that quite specific partial pressures of oxygen must be maintained for optimal aerobic growth. Thus, Biedermann et al (1967) have found 3 to 5 mm Hg partial pressure of oxygen optimal for Rsp. rubrum whilst excess aeration reduced growth. Optimal growth of R. palustris certainly did seem to occur under semi-aerobic conditions although this did allow some pigment synthesis.

The branching and knobbly morphology obtained with vigorous aeration closely resembled that observed by Van Niel (1944) using carbon sources such as caproate and crotonate during photosynthetic growth. Pfennig (1969) noted the tendancy of R. acidophila to form irregular and swollen cells. The filament-forming bacterium, Hyphomicrobium, has also been found to respond to certain environmental conditions by producing bizarre cell shapes (Tyler & Marshall, 1967). The swelling of cells at what would apparently be the division point in R. palustris (Fig. 7) is very reminiscent of the appearance of E. coli where cell division but not wall elongation has been inhibited by low concentrations of penicillin (Schwarz, Asmus & Frank, 1969; Burdett & Murray, 1974). Whether the formation of these cell types in R. palustris is as a result of abnormal or inhibited cell division is not known.

During the course of 12 years of studying non-sulphur purple bacteria Van Niel (1944) found all cultures to be motile. Generally, it was only the capsule-forming organisms that seemed to be poorly motile. Although it does not produce capsular material, R. palustris strain C1 responds to some environmental condition to become non-motile. This lack of motility is also correlated with a lesser extent of holdfast synthesis, although cells in this condition appeared to have an otherwise normal morphology. Although the trigger for motility was never determined it is interesting to note that Pfennig (1969) states that motility of R. acidophila is dependent on the presence of calcium ions. Although motility in Proteus vulgaris has been found to be naturally temperature-sensitive (McGroarty, Koffler & Smith, 1973) the increase of incubation temperature of R. palustris was not responsible for non-motility.

The tube elongation obtained under conditions of phosphate starvation in the two strains of R. palustris demonstrated a clear similarity between the tube of this species and the stalk and filaments of other prosthecate bacteria. Schmidt & Stanier (1966) demonstrated a four-fold increase in stalk length of Caulobacter crescentus under extreme phosphate-limited growth. The reproductive filaments of both Hyphomicrobium (Hirsch, 1974) and Rm. vannielii (Dow & Whittenbury, in preparation) respond to the phosphate-limited growth conditions by elongating. The reason for lack of phosphate stimulating elongation of these appendages is not clearly understood, although control of stalk synthesis in Caulobacter does appear to be related to intracellular levels of cyclic nucleotides (Schmidt & Samuelson, 1972). The fact that photosynthetic membranes are synthesised in the ends of elongated tubes of R. palustris indicates that the temporal control of photosynthetic membrane synthesis and wall growth must be by independent mechanisms.

R. palustris strain 1e5 normally has a very short tube compared with other isolates such as strain C1. Indeed, Bosecker, Drews &

Tauschel (1972) assume it to have no tube at all and on this basis Tauschel & Hoeniger (1974) incorrectly generalise that R. palustris resembles R. acidophila in producing sessile buds. During phosphate-limited growth R. acidophila does not form a tube between mother and daughter cells and therefore produces truely sessile buds. R.palustris strain 1e5 does respond to phosphate limitation by forming elongated tubes suggesting that it might have an altered mechanism controlling tube elongation.

The development of an anaerobic bag system for incubation of Rhodospirillaceae had several advantages. It was cheap (particularly if bags were reused), did not waste space, did not involve explosive gasses or high vacuums and produced uniformly pigmented colonies of the organisms in very short times relative to aerobic cultures. Probably the greatest advantage was gained in this last respect with Rm. vannielii. Contradictory reports exist concerning the ability of Rm. vannielii to grow aerobically. Pfennig (1967) and Carr (1969) both state that it is an obligate anaerobe. However, Pfennig later reported the aerobic growth of six strains of Rm. vannielii (Pfennig, 1969). Even more recently Staley (1974) has stated that Rm. vannielii cannot be grown aerobically whilst Pfennig & Truper (1974) claim in the same manual that all Rhodospirillaceae can grow at least microaerophilically. Whether these apparent contradictions reflect strain differences amongst Rm. vannielii isolates is not clear. If this is the case the anaerobic bag would be invaluable for growth of the obligate anaerobic strains.

Probably the major advantage of the anaerobic bag technique over the conventional agar deep method is that selected colonies may easily be picked off for sub-culture. Although the colonies are almost certainly growing photoheterotrophically on the media used, the fact that K_2CO_3 is used with the NaOH overcomes any difficulties in culture that might

arise from absorption of carbon dioxide by the alkali.

Thus there are a wide variety of environmental conditions which can affect either the rate of growth or mode of development, or both, in R. palustris. However, a careful choice of nutrient medium and incubation temperature, light intensity and oxygen partial pressure does seem to allow relatively predictable patterns of growth either in liquid media or by use of agar plates in anaerobic bags.

SECTION II: STUDIES ON THE CELL CYCLES OF SOME BUDDING RHODOSPIRILLACEAE

A: MORPHOLOGY AND ULTRASTRUCTURE OF R. ACIDOPHILA AND R. PALUSTRIS

SECTION IIA: MORPHOLOGY AND ULTRASTRUCTURE OF R. ACIDOPHILA AND R. PALUSTRIS

INTRODUCTION

A comparison of the morphological properties of R. acidophila, R. palustris, R. viridis and Rm. vannielii allows them to be set out as a gradient of increasing complexity of differentiation (Fig. 1, p.21). The morphological and ultrastructural development of Rm. vannielii has already been studied in some detail by Dow (1974), but morphological details of the cell cycle in R. acidophila and R. palustris have not been so completely documented. No complete sequences of shadowed or negatively stained R. acidophila have been published whilst those of R. palustris were restricted to cells in the process of tube elongation (Whittenbury & McLee, 1967). Similarly the ultrastructural changes that accompany development in these two organisms have not been set out as complete sequences through the full division cycles.

Tauschel & Hoeniger (1974) examined both morphology and ultrastructure of R. acidophila cells but observations were never considered in the context of the cell division cycle. Their most significant observations were the demonstration of peripheral layers of photosynthetic membrane lamellae, of fine surface features on the cell wall and of bundles of flagella at cell poles.

Some ultrastructural aspects of R. palustris were considered by Whittenbury & McLee (1967) where it was first shown that the tube of the organism was void of photosynthetic membranes. However, they did not show sequences of sections of complete cells through the division cycle. Neither Tauschel & Drews (1967) nor Solov'eva & Fedenko (1970) presented any electronmicrographs showing a longitudinal section of a complete cell. Tauschel & Drews (1967) attempted to establish the pattern of de novo photosynthetic membrane synthesis when R. palustris cultures were

transferred from aerobic to photosynthetic conditions. Although complex two- and three-dimensional models were presented to explain the origin of membrane structures these origins were not related to specific locations within the cells and therefore did not contribute very much to our knowledge of the normal cell cycle of R. palustris. Solov'eva & Fedenko (1970) made a brief ultrastructural comparison of wild-type R. palustris and a non-photosynthetic green mutant. Neither the aerobically grown wild-type nor the mutant under any conditions possessed photosynthetic membranes within the cells.

Thus the main aim here was to present complete sequences of morphological and ultrastructural development as revealed by the electron microscope. At the same time, this would of course allow the investigation of finer details of cell ultrastructure. In order to construct the developmental sequences of R. acidophila it was necessary to compare the apparent shape of cells with those observed during growth in slide culture. Far greater confidence could be had in the sequences of R. palustris. By shadowing samples of synchronous cultures at different times or embedding synchronous cultures after known periods of development large numbers of morphologically similar cells of known age within the cell cycle could be examined.

MATERIALS AND METHODS

Cultivation of cells. R. acidophila and R. palustris strain 1C were those isolates used in Section I. R. acidophila was grown photosynthetically on pH 5.8 PM medium. This contained (g/l): sodium pyruvate, 1.5; sodium hydrogen malate, 1.5; NH_4Cl, 0.5; NaCl, 0.4; $MgSO_4.7H_2O$, 0.4; $CaCl_2$, 0.05; and for solid medium, Bacto-agar (Difco), 15.0.

The pH was adjusted to 5.8 with KOH before autoclaving at 121° for 15 min. After sterilisation 50 ml of sterile 0.1 M phosphate buffer , pH 5.8, were added per litre of medium. Inoculated culture flasks were gassed with oxygen-free nitrogen (see Section I, p. 30) and incubated at 30° with illumination at about 1000 lux.

PAYE medium for R. palustris contained (g/l): sodium pyruvate, 1.5; sodium acetate (anhydrous), 1.5; NH_4Cl, 0.5; $MgSO_4.7H_2O$, 0.4; $CaCl_2$, 0.05; yeast extract (Difco), 1.0; and for solid medium, Bacto-agar (Difco), 15.0. The pH was adjusted to 6.9 with KOH and, after autoclaving at 121° for 15 min, 50 ml of sterile 0.1 M phosphate buffer, pH 6.9, were added per litre of medium. Aerobic cultures of R. palustris were obtained by incubating inoculated PAYE medium in cotton wool-plugged, foil-wrapped shake flasks at 30° on an orbital shaker. The cells from a second aerobic sub-culture were used for the ultrastructural studies. Photosynthetic cultures were incubated in oxygen-free nitrogen gassed flasks (see Section I, p. 30) at 36° with about 4,000 lux illumination.

Selection of synchronous cells. Synchronous populations of R. palustris swarmers were selected by a modification of the differential sedimentation method of Mitchison & Vincent (1965). The method will be described in detail in the Materials and Methods of Section IIB.

Slide culture. The appropriate nutrient agar for slide culture of organisms was boiled in a water bath for 30 min under a continuous flow of oxygen-free nitrogen. Phosphate buffer was not added until the agar had cooled to about 70°. Two drops of agar were placed on a microscope slide in a sandwich box which was continuously gassed with oxygen-free nitrogen. The agar was covered with a sterile coverslip in order to produce a thin, flat film of agar. After 5 to 10 min the coverslip was carefully removed and the agar inoculated with a drop of culture. A fresh sterile coverslip was placed on the agar and sealed onto

the slide with a mixture of petroleum jelly and paraffin wax. Examination of the slide culture was commenced as soon as the cells had stopped moving.

Light microscopy. Phase contrast light microscopy of slide cultures of R. acidophila was carried out on an Olympus EHT microscope with PM-6 camera, the whole apparatus being incubated at 30° and the slide being illuminated at about 1,000 lux. For light microscopy of R. palustris slide cultures were incubated at 34° on a Leitz heating and cooling stage 80 (E. Leitz (Instruments) Ltd, Luton, Beds). The stage was fitted to a Leitz Orthoplan microscope equipped with a Heine phase contrast condenser and a Leitz Orthomat camera. The slide culture was illuminated at approximately 4,000 lux. Photomicrographs were taken on Panatomic-X film (Kodak Ltd) which was developed with Contrast FF developer (Ilford Ltd) and printed on Ilfobrom grade 5 paper.

Electron microscopy. Bacterial cells for negative-staining and shadowing were dried onto Formvar support films on electron microscope grids. Negative-staining of whole cells was either with 0.5% uranyl acetate or 1.0% potassium phosphotungstate (pH 7.0). Cells were shadowed at an angle of approximately 35° with gold-palladium alloy in an AEI MC9 vacuum coating unit. For sectioning, cells were centrifuged and washed using a Quickfit Micro-centrifuge (Quickfit Instrumentation, James A. Jobling, Stone, Staffs) and fixed using 1% osmium tetroxide (Ryter & Kellenberger, 1958). After resuspending the cells in water agar and cutting into 2 mm cubes, cell pellets were stained with 0.5% uranyl acetate, dehydrated through a series of alcohols, transferred to propylene oxide and finally embedded in Araldite resin. The resin was freshly prepared from Araldite CY212 (resin), 4.9 g; dodecenyl succinic anhydride (hardener), 4.9 g; dibutyl phthalate (plasticiser), 0.075 g; benzyl dimethylamine (accelerator), 0.175 g. Pellets were allowed to polymerise at 60°for at least 3 d. Sections were cut with glass knives

on a Reichert OmU2 ultramicrotome.

All preparations were examined on an AEI Corinth 275 electron microscope at an accelerating voltage of 60 KV. Electronmicrographs were taken on Ilford 70 mm line film, N4E50, which was developed with Phenisol (Ilford Ltd) and printed on Kodak bromide paper.

RESULTS AND DISCUSSION

R. acidophila

The morphological and ultrastructural cell cycles of R. acidophila were constructed using individuals from heterogencous populations. Such heterogeneous populations when examined as gold-palladium shadowed preparations revealed cells of varying length (about 2 to 6 µm) and about 1 to 1.5 µm wide (Fig. 19). Although the smallest cells were a simple ovoid shape longer ones had progressively larger buds on one end. Small rosettes of cells were also occasionally observed.

When a slide culture of R. acidophila was followed it was possible to construct the cell division cycle of the organism (Fig. 20) exhibiting the developmental sequence first described by Pfennig (1969). This confirmed that the reproduction of R. acidophila was by budding directly off a mother cell and that further budding would continue from the poles of the cells which had previously been the division plane.

Using this sequence of morphogenesis it was possible to set out members of a uranyl acetate negative-stained heterogeneous population into some form of a developmental sequence (Fig. 21). Morphogenesis may best be considered starting at the newly released daughter cell (Fig. 21a). This ovoid cell appeared to be completely void of extracellular features. In fact, it is only under certain growth conditions (such as with low levels of calcium; Pfennig, 1969) or after high sheer manipulations that

Fig. 19. Gold-palladium shadowed preparation of a heterogeneous population of R acidophila. Bar represents 5 µm.

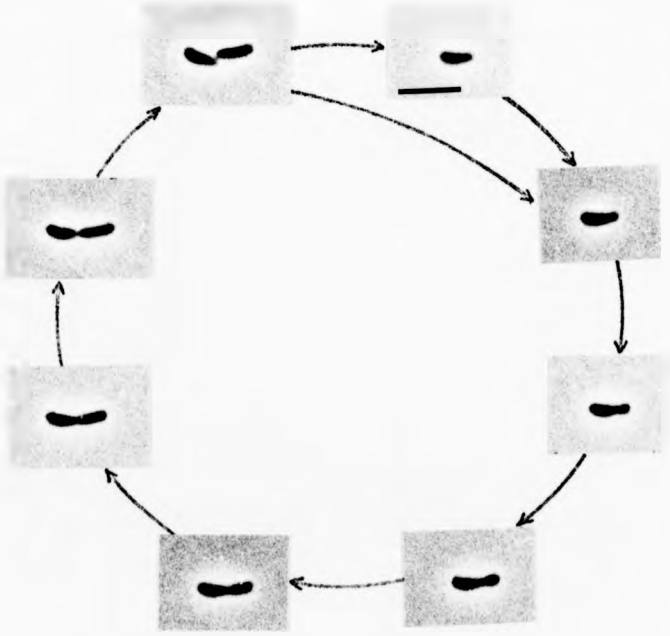

Fig. 20. Division cycle of R. acidophila constructed from phase contrast photomicrographs of a slide culture. Bar represents 5 µm.

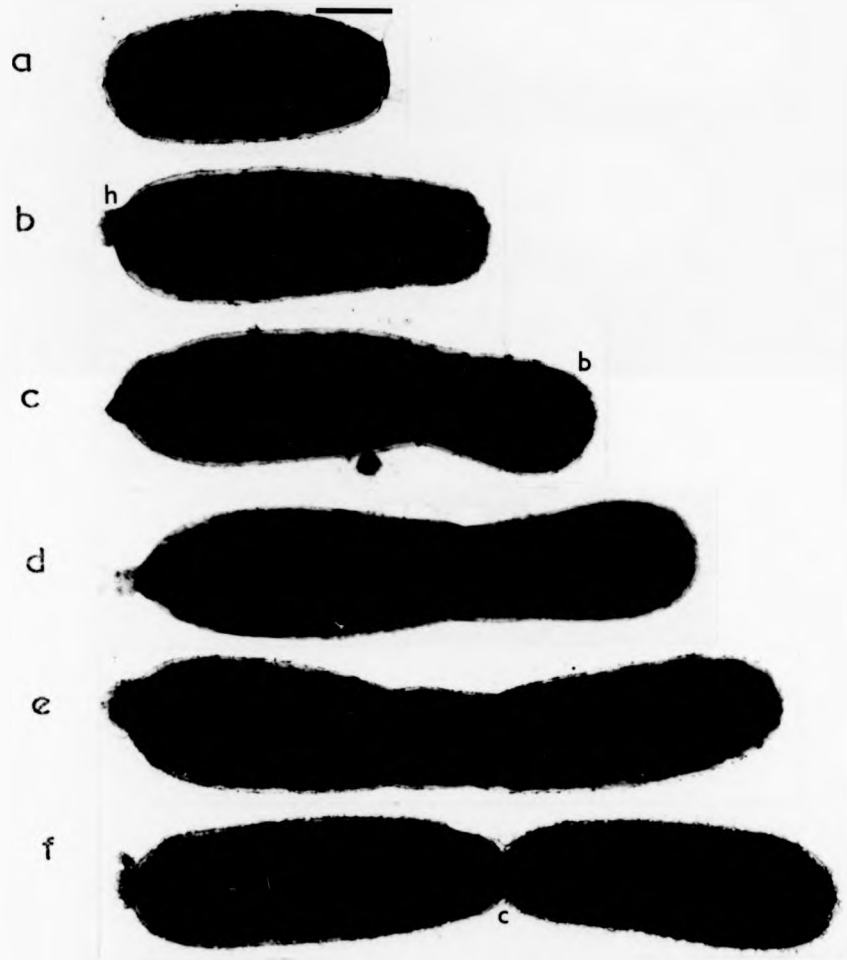

Fig. 21. Electronmicrographs of stages of morphogenesis in R. acidophila, negative-stained with 0.5% uranyl acetate. h, holdfast; b, bud; c, division constriction. Bar represents 0.5 µm.

such non-motlie daughter cells are seen. At the pole distal to that at which division occurred the daughter may bear a sub-polar bundle of flagella. These flagella were observed most clearly in shadowed preparations on the rare occasions that motile cultures were obtained (Figs 22 & 23). The fact that R. acidophila produces a bundle of flagella was first reported by Tauschel & Hoeniger (1974). Since flagella could be seen in a light photomicrograph presented by Pfennig (1969) it would seem that at least one of his strains was also producing flagellar bundles.

Cells which were starting to produce buds were always non-motile. Instead, a holdfast was synthesised at the same pole from which the flagella were released. Thus in Fig. 21b-f a holdfast was seen at the opposite pole to the growing daughter bud. In light photomicrographs and negative-stained electronmicrographs the bud was clearly seen to be derived directly from the mother cell and gradually enlarge until a symmetrical dumbell shape was produced (Fig. 21b,c). Just prior to division by constriction (Fig. 21f) daughter cells sometimes produced a new sub-polar bundle of flagella (Fig. 22). Thus division gave rise to two morphologically dissimilar cells; one bearing a holdfast and the other possibly with a bundle of flagella. Both progeny would continue to develop buds at the division poles, so at division the mother cell might be any number of generations old whilst the swarmer was ALWAYS only in its first generation. This phenomenon of ageing of cells is common to ALL bacteria that divide by budding and will be discussed at a later stage in this thesis.

Under adverse conditions of growth unwashed negative-stained preparations of R. acidophila exhibited a clearly defined capsule surrounding the cells (Fig. 24). The composition or frequency of appearance of this material is not known, although an extensive, but not so clearly defined, fibrillar capsule has also been observed by Tauschel & Hoeniger (1974).

Fig. 22. Sub-polar flagella on a dividing R. acidophila cell shadowed with gold-palladium. Bar represents 1 µm.

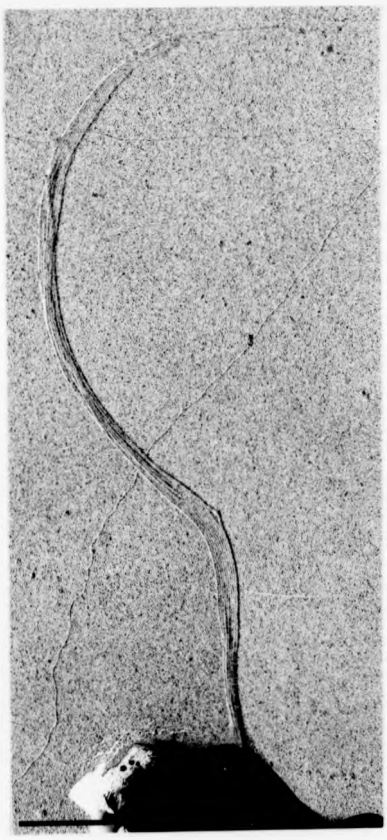

Fig. 23. Gold-palladium shadowed flagellar bundle of R. acidophila. Bar represents 0.5 µm.

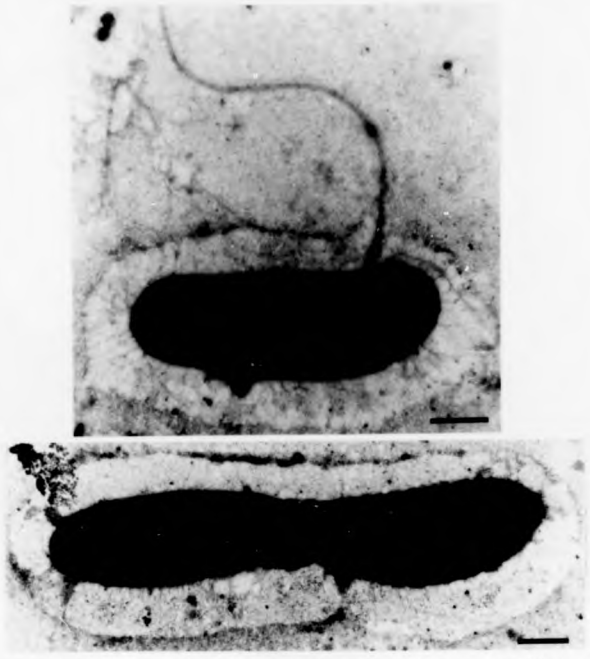

Fig. 24. Unwashed preparation of R. acidophila, negative-stained with 0.5% uranyl acetate. Bars represent 0.5 µm.

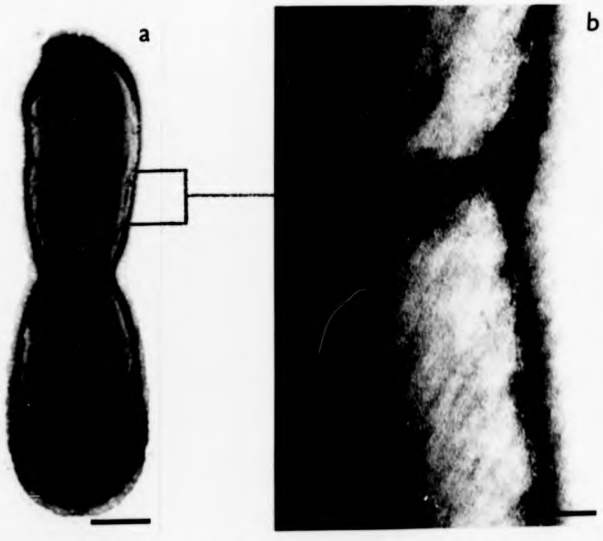

Fig. 25. Cell surface striations on R. acidophila revealed by negative-staining with 1% potassium phosphotungstate. a, bar represents 0.5 µm; b, bar represents 0.05 µm.

Tauschel & Hoeniger (1974) also noted that formaldehyde-fixed, negative-stained preparations of R. acidophila revealed a delicate surface pattern on the cell wall. The whole cell surface was covered with 9-11 nm diameter rings, of low contrast, arranged in a helical pattern. The only such ultrastructural pattern that could be distinguished on the surface of the strain of R. acidophila studied here was a feint striated appearance of the surface of a cell which had been fixed with formaldehyde and stained with potassium phosphotungstate (Fig. 25a,b). This was more reminiscent of the surface pattern detected by DeBoer & Spit (1964) after preparations of an unnamed photosynthetic bacterium using a special replica shadowing technique.

The most striking feature of transverse ultrathin sections (Fig. 26) was that the lamellar photosynthetic membranes completely encircled the cytoplasm in that plane. In this way R. acidophila resembled Rm. vannielii (Conti & Hirsch, 1965) very closely. Both transverse and longitudinal sections (Figs 26 & 27) showed the cytoplasm of R. acidophila to contain a rather irregular shaped nuclear material. Deposits of storage material, probably poly-β-hydroxybutyrate (Pfennig, 1969), were often seen within the cells.

Longitudinal sections showed continuity of photosynthetic membranes over a major part of the cell periphery although some gaps were apparent (Fig. 27a-d). Although mother and daughter cell membranes seemed to be continuous during bud formation (Fig 27b,c) a gap became apparent when cell division was about to occur (Fig. 27d). It is probable that this gap in photosynthetic membranes gave rise to the small translucent area between dividing cells, under phase contrast microscopy (Fig. 20). Exactly how R. acidophila produces a specific break in photosynthetic membranes just prior to division is not known. This problem is not encountered in R. palustris or Rm. vannielii where daughter membranes are synthesised de novo and the tube or filament between mother and daughter cells is void

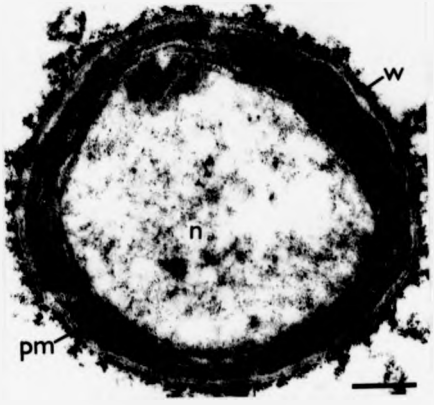

Fig. 26. Transverse ultrathin section of R. acidophila. w, cell wall; pm, photosynthetic membranes; n, nuclear material. Bar represents 0.1 µm.

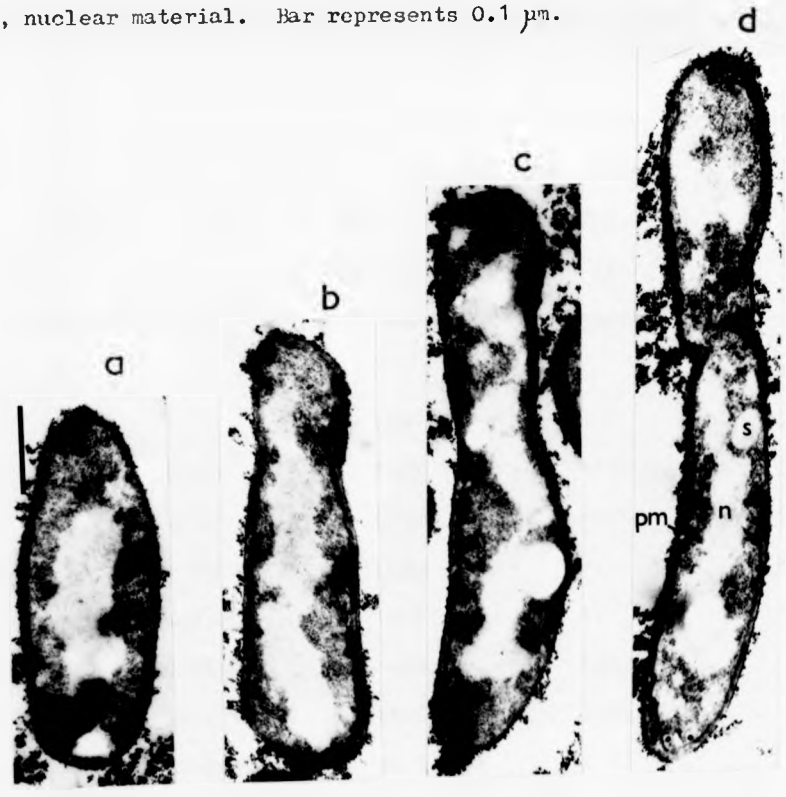

Fig. 27a-d. Developmental sequence of R. acidophila reconstructed from longitudinal thin sections. pm, photosynthetic membranes; n, nuclear material; s, storage material. Bar represents 0.5 µm.

of membranes (see next sub-section and Whittenbury & McLee, 1967; Low, 1974).

There was often observed some disturbance of the pattern of membrane layers in R. acidophila. As a result of such distortion, the cell in Fig. 28 clearly demonstrated the continuity of peripheral membranes. Very few electronmicrographs clearly exhibited the double-layering of photosynthetic membranes as shown by Tauschel & Hoeniger (1974). Perhaps this was, in part, due to the method of fixation and dehydration used. It is also important to note that in heterogeneous cultures, such as the one used here, abnormal "old" cells were examined together with the first generation cells. It has already been stated that the strain of R. acidophila used here tends to produce irregular shaped cells (Section I, Fig. 8). However, it is interesting to observe that photosynthetic membrane layers continued round the cell periphery even where the cell was severely swollen (Fig. 29). The serial sections in Fig. 29 also show that when a cell is being constricted just prior to division the photosynthetic membranes grow across the constriction compartmentalising the dividing cell.

R. palustris

When a negative-stained heterogeneous population of R. palustris was examined in the electron microscope (Fig. 30) cells were seen to be considerably smaller than those of R. acidophila. They were only two-thirds as wide (0.6 to 0.8 μm) and usually 1.5 to 5 μm in length. A number of cells, though not all, possessed flagella. The variability in length was again due to polar growth of cells as demonstrated by slide culture (Fig. 31). Slide cultures also confirmed the findings of Whittenbury & McLee (1967) that this polar growth first gives rise to a phase contrast-translucent narrowing of the cell (the tube) on the end of which develops a daughter bud. By considering this slide culture alongside representatives of shadowed synchronous populations of various ages (Fig. 32) it was possible

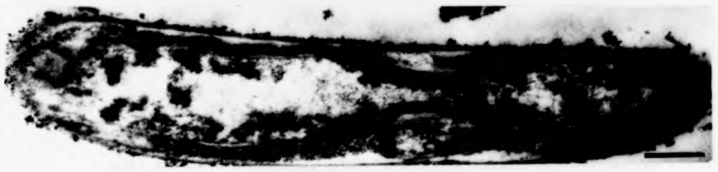

Fig. 28. Longitudinal ultrathin section of distorted R. acidophila cell showing continuity of photosynthetic membranes. Bar represents 0.2 μm.

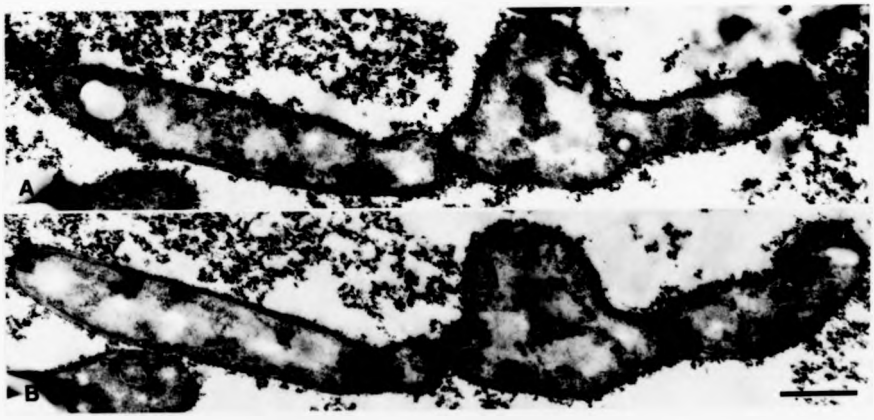

Fig. 29a & b. Serial longitudinal sections of abnormal R. acidophila cell. The swelling is bounded by membranes as is the rest of the cell. Membranes can be seen across the division constriction. Bar represents 0.5 μm.

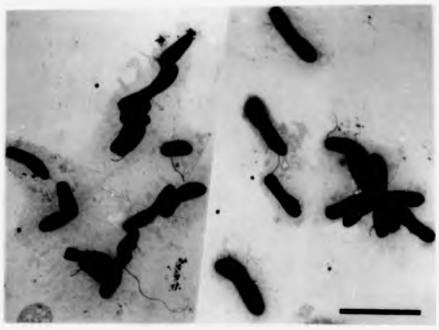

Fig. 30. Heterogeneous population of R. palustris negative-stained with 0.5% uranyl acetate. Bar represents 5 µm.

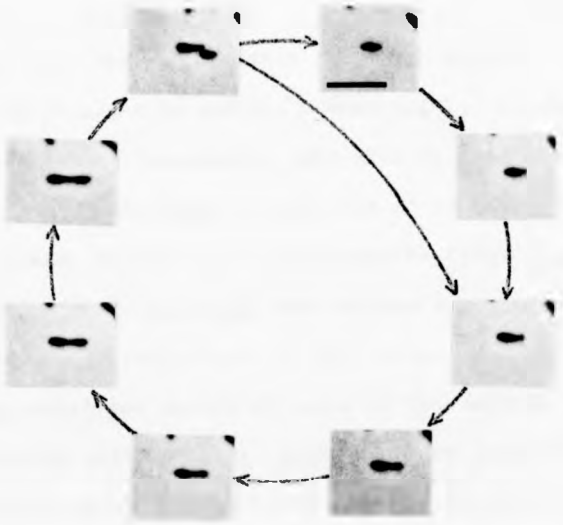

Fig. 31. Division cycle of R. palustris constructed from phase contrast light photomicrographs of a slide culture. Bar represents 5 µm.

to describe the cell cycle of R. palustris in some detail. As previously mentioned (Section I, p. 43) young daughter cells of R. palustris can, under suitable conditions, be motile by sub-polar flagella (Fig. 32a). Since dividing cells have been observed with flagella originating from beside their extreme tip, this locates the flagellate pole of the swarmer cell as being opposite that at which division occurred, that is at the end at which outgrowth would be seen. Within 1.5 to 2 h of incubation, synchronous liquid cultures of swarmers produced a holdfast at one cell pole whilst elongation of a blunt-ended tube occurred at the other pole (Fig. 32b,c). During the course of this development motility was apparently lost. After about 3 h a bud began to enlarge at the tip of the tube (Fig. 32d-f) until it was fully developed. Finally assymetrical division by constriction was observed between the mature daughter bud and the tube (Fig. 32g). This gave rise to a mother cell with holdfast and tube and a daughter cell which might be motile. Consequently, further polar growth of the mother cell immediately gave rise to a new bud on the pre-formed tube, whilst the daughter cell had to go through maturation and tube elongation phases before a bud could develop (Fig. 31). Daughter cells produced by division in R. palustris were always only in their first generation whilst mother cells could be any number of generations old. Thus R. palustris exhibited ageing of cells in the same way as R. acidophila.

Ultrathin sections of R. palustris were prepared from synchronous cultures which had been terminated at different stages of growth by addition of a drop of 1% osmium tetroxide. Examination of longitudinal sections of cells at each developmental stage permitted the construction of a sequence of ultrastructural development (Fig. 33). In contrast to R. acidophila the nuclear material of R. palustris was a more compact fibrillar body. Elongation of the nuclear material only really became apparent after about 2.5 h (Fig. 33c) and, as development proceeded, extended along most of the length of the cells. Even as the

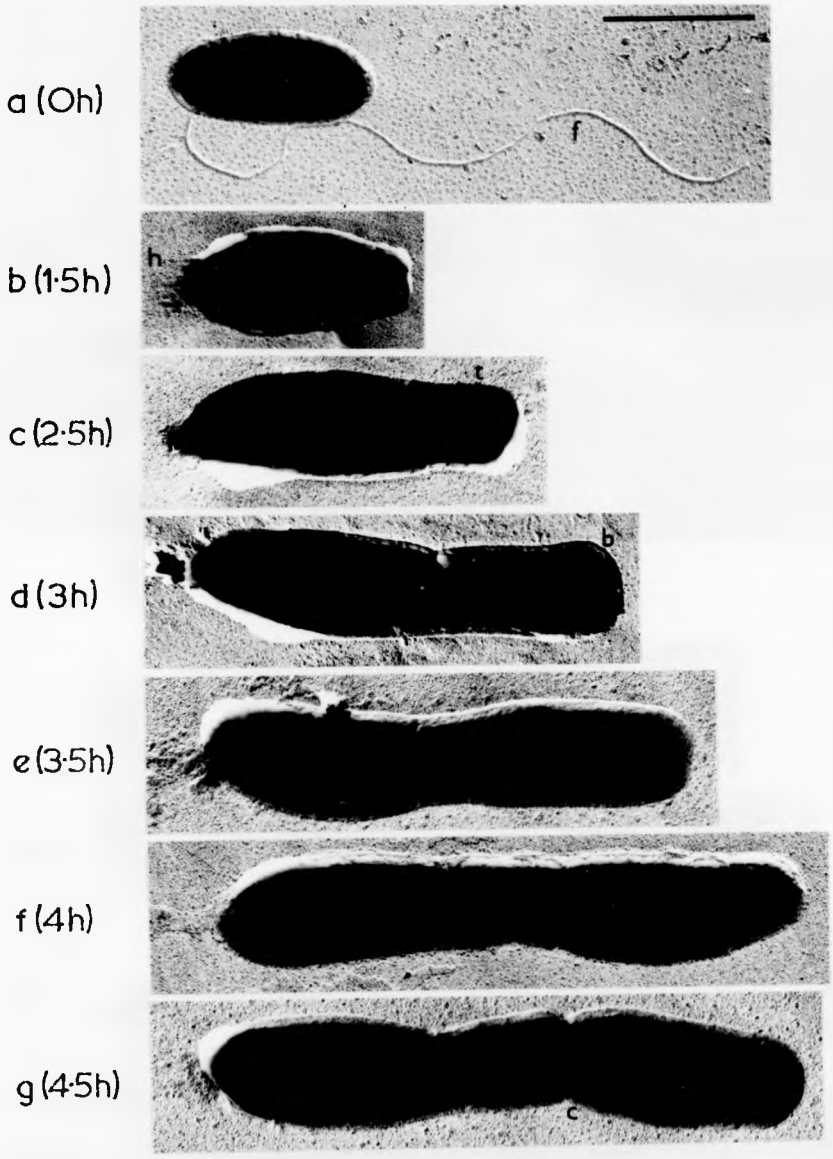

Fig. 52a-g. Electronmicrographs of stages of morphogenesis observed in a synchronous culture of A. palustris. Cells were shadowed with gold-palladium. f, flagellum; h, holdfast; t, tube; b, bud; c, division constriction. Bar represents 1 µm.

Fig. 34. Transverse section of R. palustris. w, cell wall; pm, photosynthetic membranes; n, nuclear material; m, plasma membrane. Bar represents 0.1 µm.

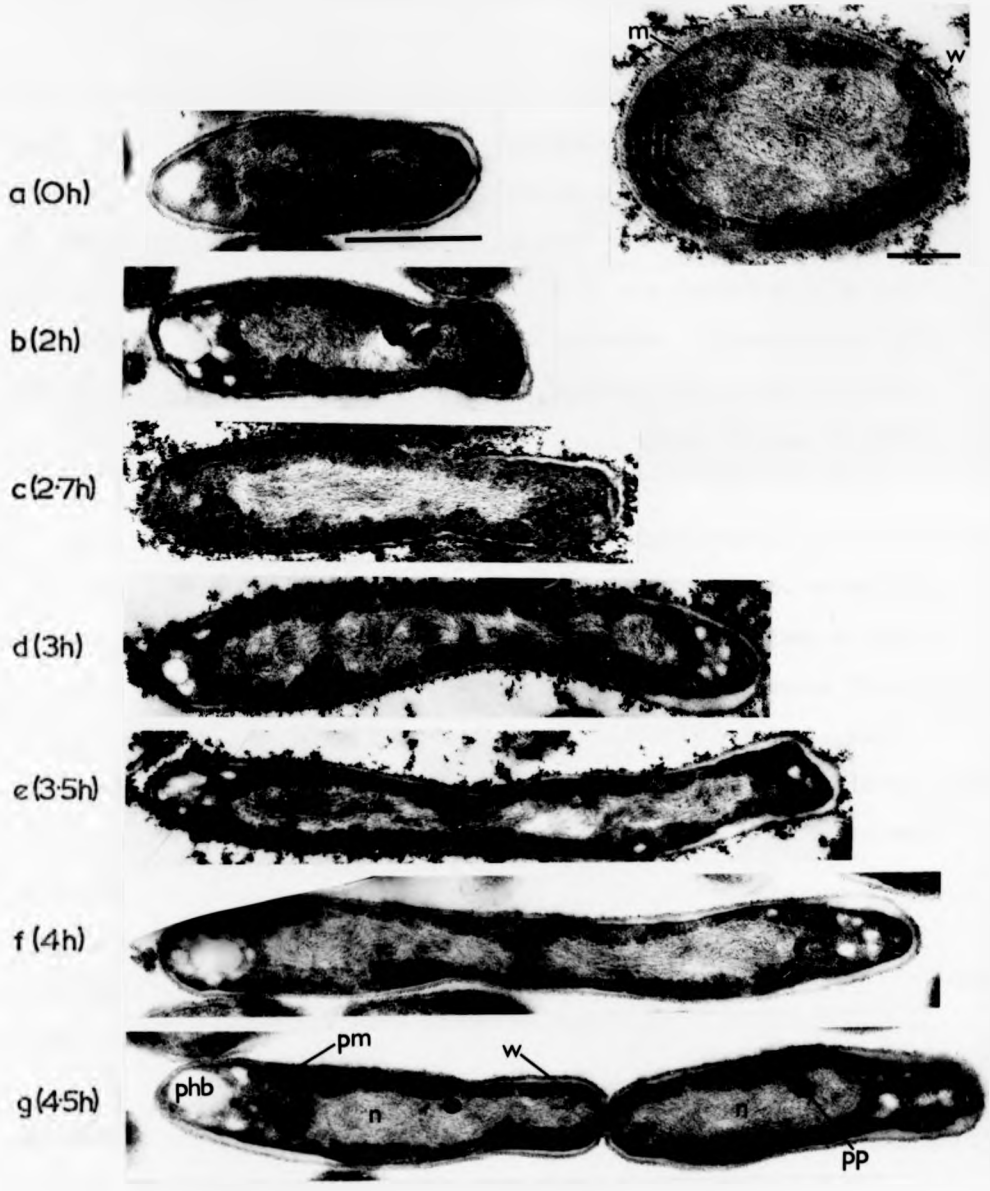

Fig. 33a-g. Ultrathin sections of cells from synchronous populations of R. palustris. pm, photosynthetic membranes; w, cell wall; n, nuclear material; phb, poly β-hydroxybutyrate; pp, polyphosphate. Bar represents 0.5 µm.

constriction which would lead to division (Fig. 33g) was beginning to form the nuclear body of the cell could still be seen to be continuous along the length of the cell (Fig. 35). A very fine strand of cytoplasm could be detected within the constriction at a very late stage of division (Fig. 36) although there was some indication that the plasma membranes had separated the contents of the two cells. The serial sections in Fig. 37 suggest that division was completed by the formation of a small break or nick in the cell wall at the constriction. Alternatively this may have been due to mechanical damage of the cell during preparation.

Within the nuclear material electron-dense bodies, probably consisting of polyphosphate, were sometimes apparent (Fig. 33a,b,g). At the holdfast pole of the cells were seen electron-transparent inclusions within the cytoplasm (Fig. 33). Inclusions were seen at the other pole of the cells only after bud formation had started.. Comparison of these electronmicrographs of R. palustris with those of other workers (Conti & Hirsch, 1965; Tauschel & Drews, 1967) indicate that these inclusions consisted of poly-β-hydroxybutyrate. The synthesis of poly-β-hydroxybutyrate by photosynthetic bacteria growing on acetate is, in fact, a quite well established phenomenon (Pfennig, 1969; Stanier et al, 1959). On some occasions the inclusions were seen to have a characteristic central circular deposit surrounded on its circumference by a number of smaller and less well defined electron-transparent circular areas (Figs 33 & 38).

It was not very common for the holdfast material to be apparent in thin sections since much of it was easily removed by washing and, in any case, the cells, which were usually non-motile, did not produce very much holdfast material. However, Fig. 39 shows the appearance of the holdfast in section. The composition of the material is not known, but Fig. 39 does indicate that it may originate directly from the outer layer of the cell wall. It is possible, then, that holdfasts may

Fig. 35. Ultrathin section of R. palustris cell showing continuity of nuclear material prior to division. Bar represents 0.2 μm.

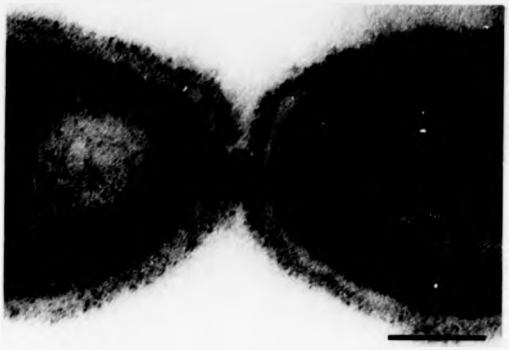

Fig. 36. Division constriction of R. palustris. Chromosomes have been separated but the daughter cell has not been released. Bar represents 0.1 μm.

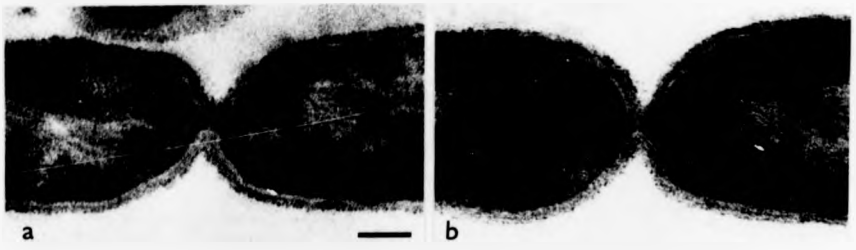

Fig. 37a & b. Serial sections through R. palustris division constriction. A nick in the cell wall is apparent in Fig. 37a. Bar represents 0.1 μm.

Fig. 35. Ultrathin section of R. palustris cell showing continuity of nuclear material prior to division. Bar represents 0.2 μm.

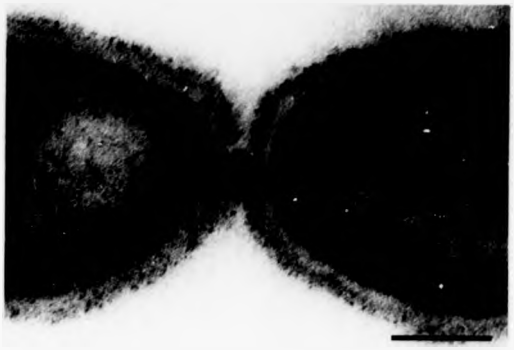

Fig. 36. Division constriction of R. palustris. Chromosomes have been separated but the daughter cell has not been released. Bar represents 0.1 μm.

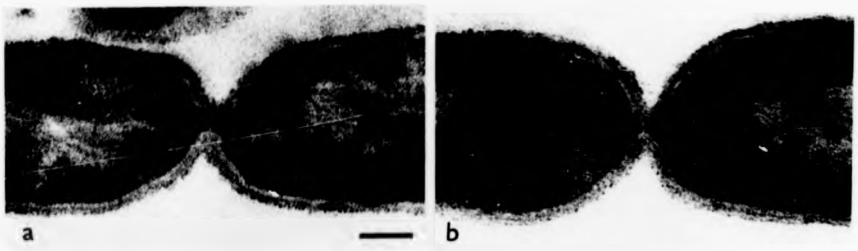

Fig. 37a & b. Serial sections through R. palustris division constriction. A nick in the cell wall is apparent in Fig. 37a. Bar represents 0.1 μm.

Fig. 38. Transverse section of R. palustris cells showing characteristic pattern of polar storage deposits (probably poly β-hydroxybutyrate). Bar represents 0.2 μm.

Fig. 39. Longitudinal section through cell pole and holdfast of R. palustris. Bar represents 0.1 μm.

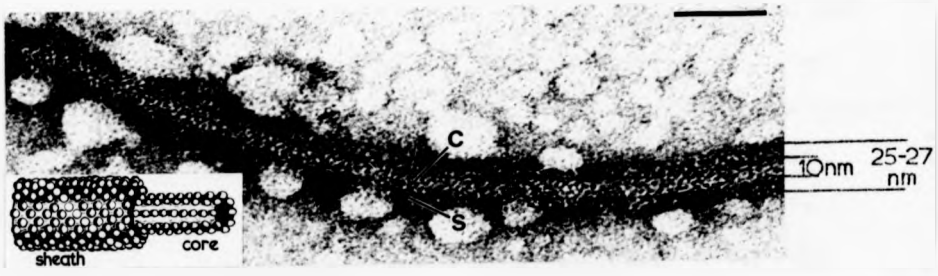

Fig. 40. R. palustris flagellum negative-stained with 2% potassium phosphotungstate, pH 7.0. c, core; s, sheath. Bar represents 0.05 μm. Insert shows diagrammatic representation of flagellar structure (Tauschel & Drews, 1970).

be a localised deposit of specialised sticky cell wall polysaccharide or lipopolysaccharide.

Flagella were never recognised in thin sections. In order to observe their ultrastructure it was necessary to examine the flagella of motile cells negative-stained with potassium phosphotungstate (Fig. 40). By this means it was possible to distinguish a central flagellar core of about 10 nm diameter surrounded by a less dense sheath giving the complete flagellar filament a diameter of about 25 to 27 nm. It was Tauschel & Drews (1970) who first showed R. palustris to have a sheathed flagellum. It was also reported that the sheath material was of a non-proteinous nature (Tauschel & Drews, 1970; Tauschel, 1970) whereas the core was composed of molecules of a characteristic flagellin (Tauschel, 1971).

Perhaps the most interesting ultrastructural feature of the division cycle of R. palustris is the appearance and location of photosynthetic membrane lamellae. The electronmicrographs presented here very clearly confirm the observation of Whittenbury & McLee (1967) that photosynthetic membranes always appear in the body of the cells and never within the tube (Fig. 33). Examination of a large number of cells from synchronous populations indicated that membranes could be detected very early during the stage of bud formation. The horseshoe shape of the membranes when viewed in transverse section (Fig. 34) explains the frequent appearance of lamellae on only one side of the cell in longitudinal sections. Serial transverse sections of a group of cells (Fig. 41) demonstrated that the gaps in the membranes at one point of the cell circumference were always maintained. This horseshoe appearance of membranes had also been seen by Whittenbury & McLee (1967). Solov'eva & Fedenko (1970) had wrongly assumed that the appearance of photosynthetic membranes in two groups of lamellae opposite each other on the cell periphery in longitudinal sections meant that they were composed of two

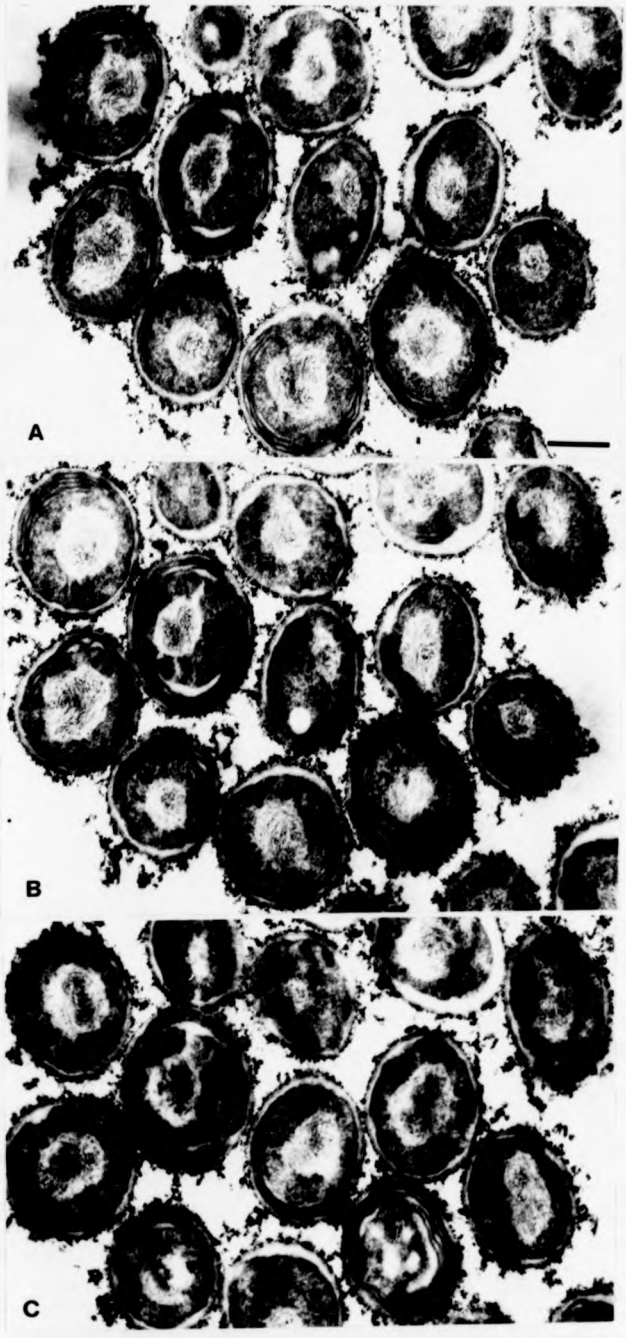

Fig. 41a-c. Serial transverse sections of R. palustris. Close examination reveals that the gap in the membrane horseshoe is always present. Bar represents 0.2 µm.

piles of symmetrically located thylakoids. Clearly they had not been aware of the appearance of membranes in transverse sections and, indeed, none were presented. Tauschel & Drews (1967) showed photosynthetic membranes in transverse serial sections of their R. palustris cells to be of a very complex nature and in some cases they even encircled the whole cytoplasm. However the cells which they studied seemed very similar in appearance to abnormal and perhaps "old" organisms (C.S. Dow, personal communication). The high degree of lamellar branching, frequently inter-leaved with broad layers of cytoplasm and periplasm, was not typical of first generation cells or young mother cells grown photosynthetically.

The only deviation from the simple layered structure that was seen in sections of cells in this study was the appearance of curious circles of membrane from which stemmed membrane layers of the normal type (Fig 42). This structure was not very frequently observed in R. palustris and it is not certain whether it served a specific function in the light absorbing system of the cell or whether it was an artefact or resulted from abnormal membrane growth.

Another phenomenon that was occasionally observed in transverse sections was the presence of finger-like projections of periplasm pointing into the centre of cells through the gap of the horseshoe membrane shape (Fig. 43). It is possible that these might be plasma membrane extentions to the nuclear material and concerned with genome replication, although they might again be explained as artefacts due to cell distortion.

Close examination of photosynthetic lamellae showed them to consist of pairs of unit membranes forming a complex of three electron-dense layers, the central layer of which was always heavily stained (Fig. 44). The appearance of this "triplet" (Murray & Watson, 1965) has similarly been found in the photosynthetic membrane lamellae of R. acidophila (Tauschel & Hoeniger, 1974), R. viridis (Drews & Giesbrecht, 1965) and Rm. vannielii (Conti & Hirsch, 1965).

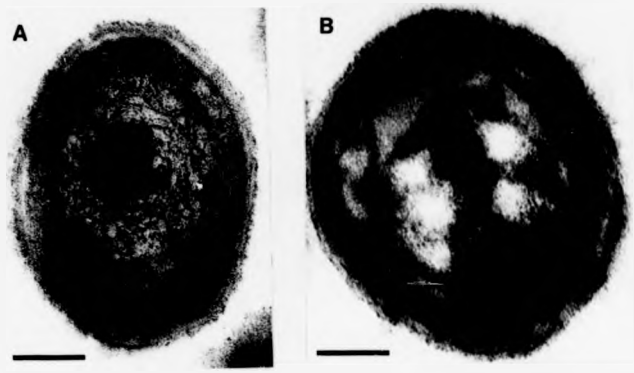

Fig. 42a & b. Transverse ultrathin sections of R. palustris showing membrane circles and ajoining lamellae.
Bars represent 0.1 µm.

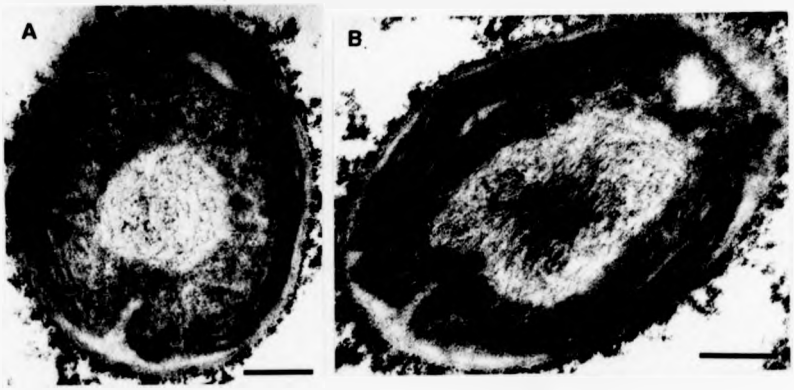

Fig. 43a & b. Transverse ultrathin sections of R. palustris showing periplasmic projection in gap of membrane horseshoe.
Bars represent 0.1 µm.

Fig. 44. Cell wall of R. palustris and underlying double-tracked photosynthetic membrane lamellae. Bar represents 0.05 µm.

Another feature that is clear from Fig. 44 is that between the photosynthetic membrane lamellae and the cell wall there is no discernable plasma membrane. Instead a single membrane is observed on the inner-facing surface of the lamellae. The change along the length of the cell from single plasma membrane to a double-layered photosynthetic membrane stack is normally apparently abrupt (Fig. 33); however, serial longitudinal sections of the mother cell shown in Fig. 45 exhibited the rare occurrence of an infolding of the plasma membrane giving evidence of a possible origin of the photosynthetic membranes. The membranes are seen to "grow" away from the tube end of the body of the mother cell and thus defines the start of the tube. Close examination of the daughter cell membranes in Fig. 33f revealed another such membrane origin (Fig. 46), again with the photosynthetic membranes "growing" away from the end of the tube. From these two examples a composite diagrammatic representation has been constructed (Fig. 47) showing how the membrane origins might define the boundaries between cell bodies and the tube. Whittenbury & McLee (1967) and Tauschel & Drews (1967) both proposed a similar infolding of the plasma membrane although the location of such infolding was not defined. On the basis of the information presented here three alternative models of how membrane layers may be derived from an infolding of a continuous plasma membrane can be proposed (Fig. 48). Further electron microscopy studies might reveal which of these models, if any, is correct.

When sections of aerobically grown R. palustris cells were examined the highly organised lamellar membrane structure was found to be totally absent (Fig. 49). In contrast to individuals from photosynthetically grown cultures, the cells frequently had large periplasmic spaces between the cell wall and a rather undulating plasma membrane. This resulted in the frequent appearance of folds and pockets of membranes both inside and outside the natural line of the plasma membrane (Figs 49b & 50).

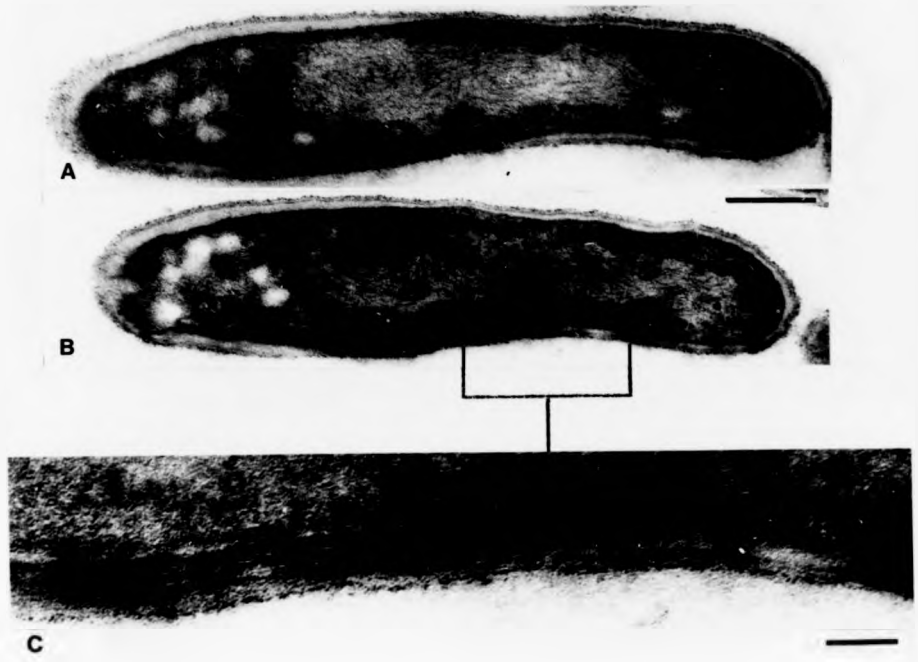

Fig. 45a-c. Membrane origin in R. palustris mother cell. a & b, serial sections showing location and depth of infolding in cell. Bar represents 0.2 µm; c, enlargement of infolding shown in Fig. 45b. Bar represents 0.05 µm.

Fig. 46. Membrane infolding of R. palustris mature bud shown in Fig. 33f. Bar represents 0.05 µm.

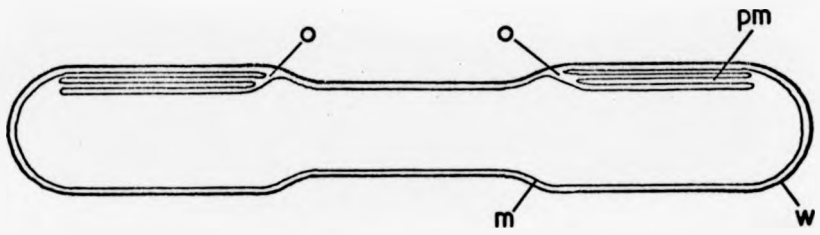

Fig. 47. Schematic mature R. palustris cell showing location and direction of development of photosynthetic membrane lamellae. w, cell wall; m, plasma membrane; pm, photosynthetic membrane lamellae; o, origin of photosynthetic membrane lamellae.

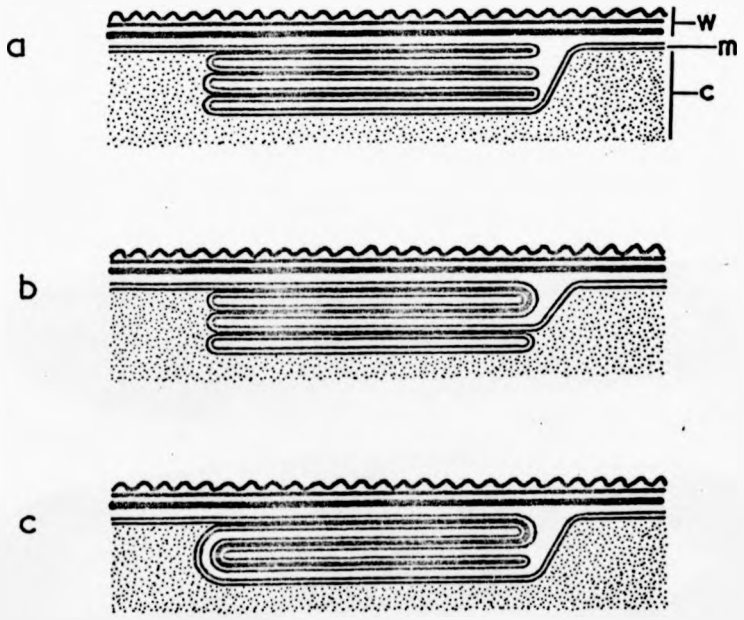

Fig. 48a-c. Models of possible membrane foldings to give triplets with an inner single membrane layer. w, cell wall; m, plasma membrane; c, cytoplasm.

Fig. 49a & b. Serial longitudinal sections of aerobically grown R. palustris cells showing lack of photosynthetic membranes. Bar represents 0.1 μm.

Fig. 50. Longitudinal thin sections of aerobically grown R. palustris cells showing plasma membrane infoldings (a) and outfoldings (b). Bars represent 0.2 μm.

However their location was never consistent, suggesting that they were not related to the photosynthetic membrane infoldings described above. Whether they resulted from excessive plasma membrane synthesis or were due to distortion by fixation and dehydration techniques is not known.

Comparison of R. acidophila, R. palustris and Rm. vannielii

From the data accumulated on R. acidophila and R. palustris a comparison of many of the relevant morphological and ultrastructural features can be made between these organisms and Rm. vannielii (Table 5). From this summary of the comparative morphology and ultrastructure it becomes evident that although the three organisms are physiologically very closely related and all have basically the same mode of growth, i.e. polar growth with budding reproduction, many of the morphological and ultrastructural details differ. A number of these differences perhaps would not be expected in a closely related group such as this. For instance, polarly and peritrichously flagellate organisms are rarely found to be related. On the other hand, the different ways in which polar growth and budding reproduction are expressed clearly demonstrates a range of increasing complexity from R. acidophila to R. palustris to Rm. vannielii and it is on this basis that the group is particularly suitable for studying morphogenesis and differentiation.

Table 5. Comparative morphological and ultrastructural properties of the budding photosynthetic bacteria.

R. acidophila	*R. palustris*	*Rm. vannielii**
swarmer 1-1.5 μm wide	swarmer 0.6-0.8 μm wide	swarmer ~1.2 μm wide
swarmer 2-3 μm long	swarmer 1.5-2 μm long	swarmer ~2.8 μm long
unidirectional polar growth	unidirectional polar growth	bidirectional polar growth
budding reproduction	budding reproduction	budding reproduction
sessile buds	bud on end of tube	bud on end of filament
division by constriction	division by constriction	division by constriction; or plug formation
branching abnormal	branching abnormal	branching "mycelial" growth normal
holdfast on mother cell	holdfast on mother cell	no holdfast
sub-polar insertion of flagella	sub-polar insertion of flagellum	peritrichous flagella
flagella in bundles	single flagellum	many flagella
flagella not sheathed	flagellum sheathed	flagella not sheathed (?)
lamellar photosynthetic membranes (p.m.)	lamellar p.m.	lamellar p.m.
p.m. around poles of cells	p.m. never around cell poles	p.m. may or may not be around poles of cells
p.m. around complete transverse circumference	p.m. forms horseshoe around transverse circumference	p.m. around complete transverse circumference (moderate light)
vegetative cells only	vegetative cells only	some strains form exospores
may have capsule	no capsule apparent	capsule only apparent on exospore

*, data obtained from Vatter, Douglas & Wolfe (1959), Duchow & Douglas (1949), Conti & Hirsch (1965), Trentini & Starr (1967) and Dow (1974).

SECTION II: STUDIES ON THE CELL CYCLES OF SOME BUDDING RHODOSPIRILLACEAE

B: SOME PROPERTIES OF SYNCHRONOUS R. PALUSTRIS CULTURES

SECTION IIB: SOME PROPERTIES OF SYNCHRONOUS R. PALUSTRIS CULTURES

INTRODUCTION

In the study of the division cycle of organisms it is essential that large numbers of cells can be obtained which develop in the same way at the same time. In bacteria that divide by symmetrical binary fission synchronous populations of cells will usually be all of the same age since no individuals become more than one generation old. A budding bacterium in the process of developing its bud might have produced any number of buds before and therefore may be less than one generation old or may be any number of generations old. It is important to note, therefore, that it is possible to have synchronous growth of budding cells which are of different ages.

Principally there are two types of methods of obtaining synchronous cells: phasing methods and selection methods (Halvorson, Carter & Tauro, 1971). A wide variety of phasing techniques have been employed, each involving changes in the environment to effect synchronisation of a population of cells. Such manipulations of temperature, nutrients, illumination or end point of growth are almost certain to result in abnormal or unbalanced growth and there are, therefore, considerable objection to their use for biochemical studies. Selection methods of synchronisation offer a wide range of techniques for physical separation of physiologically identical cells from an exponential culture, usually with minimum disturbance to the cells. Maruyama & Yanagita (1956) were first to describe a successful method of selecting synchronous bacterial populations. This was by filtration through a paper pile in a filter press. A similar method using a combination of paper and glass fibre filters has been described more recently by Sargent (1973). Selection of synchronous cells by adsorption onto binding membranes and release by growth was

pioneered by Helmstetter & Cummings (1963, 1964). Although several workers have used this method with E. coli B/r, little success has been achieved with other strains or organisms. The third major selection method for obtaining synchronous cells is the differential sedimentation method by centrifugation. This method was first described by Maruyama & Yanagita (1956) and then further refined by Mitchison & Vincent (1965). Here, separation depends on cells at different developmental stages having different sedimentation velocities.

Several workers have studied synchronous populations of organisms which are physiologically or morphologically related to R. palustris. A non-budding member of the Rhodospirillaceae, Rhodopseudomonas spheroides, has been synchronised by the stationary phase method of Cutler & Evans (1966). Perhaps this phasing method of synchronisation was an unfortunate choice when the timing of enzyme synthesis and adaptation was being studied (Ferretti & Gray, 1967,1968).

Caulobacter, which divides by assymetrical binary fission, giving rise to a motile swarmer and non-motile stalked cell (Stove & Stanier, 1962), is morphogenetically quite similar to R. palustris (see Fig. 1, p. 21). Numerous workers have studied synchronous cultures of various species of Caulobacter. A centrifugation method of synchronisation has been described where stalked cells were found to have the lowest sedimentation velocity of cells from heterogeneous populations (Stove & Stanier, 1962; Poindexter, 1964). Shapiro & Agabian-Keshishian (1970) used a system involving multiple steps of centrifugation. Schmidt & Stanier (1966) were able to select Caulobacter swarmers after filtration of a culture through a 4 mm cellulose fibre layer. Degnen & Newton (1972a) described a novel method of cell synchronisation which depended on the adsorption of stalked cells onto large Petri dishes by means of their holdfasts. Either released cells (swarmers) or adsorbed stalked cells

could be selected. Finally, Staley & Jordan (1973) selected Caulobacter swarmers which were released from a stalked cell culture adsorbed onto glass beads.

The filament forming, non-photosynthetic, budding organism, Hyphomicrobium, has now been studied in synchronous culture. Moore & Hirsch (1973a) obtained synchronous cultures of Hyphomicrobium strain B-522 by a combined centrifugation and filtration technique. Again the method was designed to select the small swarmer cells. Brandon & Norris (1972) described a continuous-flow synchronisation method which they used for H. vulgare, where mother cells with filaments were supported on glass beads by natural adhesion and swarmers were continuously released.

Selection of synchronous R. palustris cells has only once been described, by Whittenbury & McLee (1967). This was achieved by sucrose density gradient centrifugation using the method of Mitchison & Vincent (1965). Unfortunately, data concerning the degree of synchronisation was not presented. However, the synchronous cultures did allow the authors to confirm the uniformity of the basic developmental pattern in R. palustris swarmers and conclude that the organism reproduced by budding.

The only other budding Rhodospirillaceae that has been grown in synchronous culture is Rm. vannielii. Dow (1974) was able to obtain good yields of well synchronised swarmer cells by passing a heterogeneous culture through a column packed with glass wool and glass chromatography beads. All other cell types were probably retained by a combination of adsorption and filtration. Since R. palustris does not produce large networks of branched filaments as does Rm. vannielii it is not surprising that attempts at obtaining synchronous cultures using glass wool columns have been unsuccessful. Like Caulobacter, R. palustris has a sticky holdfast and, therefore, it might be anticipated that synchronisation could be achieved by adsorption methods. However, since populations of

swarmer cells had previously been obtained by sucrose density gradient centrifugation this method was pursued and refined for the strain of R. palustris used in this study. Using selected swarmer populations it was then possible to examine the properties of the synchronous cells and determine the timing and occurrence of events during the growth of the cells. In this way the properties of the cell cycle of R. palustris could be defined in detail.

MATERIALS AND METHODS

Cultivation of cells. R. palustris strain C1 was grown photosynthetically in PAYE medium (Section IIA, p. 61). A 1% inoculum was used for 100 ml liquid heterogeneous cultures in 250 ml flasks, gassed with oxygen-free nitrogen (Section I, p. 30). Cultures were incubated at 34° with approximately 4,000 lux illumination and generally used after 22 h incubation when they were in the mid-exponential phase of growth.

Slide culture and microscopy was carried out as described in Section IIA (p. 61 & 62).

Selection of synchronous cells. Cultures were synchronised by a modification of the sucrose density gradient sedimentation method of Mitchison & Vincent (1965). The cells from 75 to 100 ml of a mid-exponential culture were concentrated 25-fold by centrifugation and layered onto a 30 ml linear sucrose gradient (7.5 to 15%, sucrose in PAYE). Centrifugation was for 9 min at 3,300 rev/min (approximately 4,200 x *g*.) using the swingout rotor of an MSE Multex centrifuge (MSE, Crawley, Sussex). Swarmer cells were harvested from the uppermost layers of the band of cells, about one-third to half of the way down the gradient (Fig. 51).

In an initial experiment the concentration of sucrose was

estimated in fractions of the gradient by measuring the refractive index of the samples at 25° and converting the results to equivalent sucrose concentration (Fig. 51). The method was found to be rather inaccurate, particularly as the presence of cells affected the refractive index. Whilst collecting fractions for the refractivity measurements the optical density was measured by passing the collected gradient through the flow-through cuvette of a Cecil Model CE202 spectrophotometer (Cecil Instruments Ltd, Cambridge) which was connected to a Servoscribe recorder (Smiths Industries Ltd, Wembley, Middx).

Cell counts. Liquid synchronous cultures for viable cell counts were always prepared as swarmers from sucrose gradients, diluted 10-fold in PAYE. 0.1 ml samples from the cultures were serially diluted in phosphate buffer and duplicate spread plates prepared on PAYE agar. Plates were incubated at 30°, under illumination in the anaerobic bag described in Section I (p. 32), for about 4 d.

Total cell counts of synchronous cultures were made using a Thoma 0.02 mm deep bacterial counting chamber (Gallenkamp Ltd, London). A synchronous culture was prepared using 1.5 ml of swarmers from a sucrose gradient, diluted to 10 ml with PAYE. 0.1 ml samples were removed at hourly intervals and fixed by the addition of 0.1 ml of 0.1 N HCl. After thorough mixing, the total cell counts of drops of the suspensions were estimated using the counting chamber. Each culture sample was counted two or three times independently.

Cuvette cultures. Cuvette cultures for studying synchronous populations were prepared by a modification of the method described in Section I. 3.5 ml cultures were contained in round-topped 1 cm glass cuvettes. The cuvettes were stoppered with No. 17 standard turn-over type Suba-Seals (William Freeman & Co. Ltd), syringe needles being used to release air trapped by the Suba-Seals. Each cuvette was gently gassed

for about 1 min with oxygen-free nitrogen. Between periodic optical density measurements cuvettes were incubated in a water bath at 34° with about 4,000 lux illumination.

Coulter counter analysis. The frequency distribution of particle volumes in chilled samples from liquid cultures was determined using a Model ZF Coulter counter (Coulter Electronics Ltd, Dunstable, Beds.) together with a Coulter Channelyzer and XY Recorder II. Synchronous cultures were prepared for this purpose as 10-fold dilutions of swarmers from sucrose gradients. Culture samples were diluted 100-fold in ice-cold phosphate buffer and particle counts made immediately on a subsequent 10-fold dilution of each sample in Isoton (Coulter Electronics Ltd) using a 30 μm aperture. Frequency distributions of particle volumes were accumulated in the Channelyzer over an arbitrary period of sample analysis before printing out the distributions on the Recorder.

Total particle counts were made on 0.05 ml samples of the Isoton dilutions drawn through the aperture and the mean of six counts converted to particles/ ml of synchronous culture.

Giemsa stain. Samples were taken from synchronous liquid cultures, chilled, centrifuged and washed twice with water using a Quickfit Micro-centrifuge. The pellets were resuspended in small volumes of water and an air-dried smear prepared on a cleaned microscope slide. The working solution of Giemsa stain was freshly prepared as a 10-fold dilution of commercially available stain (BDH Chemicals Ltd, Poole, Dorset) in pH 6.9 phosphate buffer. Dried smears were stained by the following treatment: absolute methanol, 5 min; 1.0 N HCl, 60°, 15 min; distilled water, rinse; dilute Giemsa stain, 37°, 18 h; distilled water, rinse; phosphate buffer, pH 6.9, 1 min. Stained preparations were gently blotted, protected with sealed coverslips and examined by phase contrast microscopy.

Incorporation of ^{32}P-phosphate. Synthesis of DNA was presumptively estimated by measuring incorporation of ^{32}P-orthophosphate (initially 94 Ci/mg P; Radiochemical Centre, Amersham, Bucks.) into alkali-resistant material, whilst alkali-soluble material was estimated as the difference between this and the total incorporation of ^{32}P. Liquid cultures were labelled with 8 μCi/ml ^{32}P-phosphate in PAYE medium containing only 1 mM phosphate buffer. Total incorporation was measured in 0.1 ml samples treated at 0°for 30 min with 1 ml of 10% trichloroacetic acid (TCA) containing 10 μg/ml bovine serum albumen (BSA). Precipitated nucleic acid was washed, on 2.5 cm Whatman GF/C filters, with cold 10% TCA, phosphate buffer, ethanol and ether and then dried. Uptake of ^{32}P into DNA was measured in duplicate 0.1 ml samples which were treated for 1 h at 37° with 1 ml of 0.4 N NaOH containing 10 μg/ml BSA. The alkali was then neutralised with 1 ml of 0.4 N HCl. Samples were finally treated with 2.5 ml of 18% TCA for 30 min at 0° and then filtered, washed and dried as before. Radioactivity on filters was estimated in 10 ml aliquots of triton X-toluene scintillation fluid using a Packard Tri-Carb scintillation counter.

Incorporation of ^{3}H-leucine. Incorporation of ^{3}H-leucine (53 Ci/mmol; Radiochemical Centre) was measured in cells which had been grown overnight in PAYE, supplemented with 50 μg/ml L-leucine. ^{3}H-leucine was added at an activity of 5 μCi/ml to synchronous and heterogeneous sub-cultures of the cells in PAYE containing only 0.02 g/l yeast extract and supplemented with 5 μg/ml L-leucine. 0.1 ml samples were treated with 1 ml of 10% TCA at 0° for 30 min and then filtered, washed with 50 μg/ml L-leucine, dried and counted as described for ^{32}P-phosphate incorporation above.

RESULTS

Selection of swarmers on sucrose gradients

Sucrose was found to be the most convenient high density substrate for density gradient centrifugation. Attempts were made to replace sucrose with Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) since this high density co-polymer offers a low osmotic pressure and low viscosity. However, unreliable results were obtained when Ficoll was dissolved in nutrient medium for preparation of gradients and it appeared that salts interfered with the co-polymerisation of the constituent molecules.

Extensive trials revealed that a linear gradient of sucrose from about 7.5% to 15% centrifuged for about 9 min at 3,300 rev/min (approximately 4,200 x *g*.) gave the most suitable separation and distribution of cell types (Fig. 51). The sucrose gradient was prepared with nutrient medium to alleviate much of the physiological shock resulting from this method of synchronisation. Osmotic shock still probably remained, but cells appeared morphologically normal in the sucrose and, as will be seen later, synchronous cultures started to increase in optical density from zero time, indicating immediate growth.

By loading such sucrose gradients with cells concentrated from about 75 ml of mid-exponential culture, up to 1.8 ml of synchronous cells at concentrations up to 1.4×10^9/ml have been obtained from the uppermost layers of cells after centrifugation. Smaller samples containing lower concentrations of cells yielded populations exhibiting better synchrony. Consequently up to 1.5 ml containing about 5×10^8 cells/ml were routinely removed from centrifuged gradients and used as synchronous populations.

An examination of synchronous cell populations revealed more than 95% of cells to be swarmers (Fig. 52). By following development of a single field of a slide culture at time intervals after inoculation

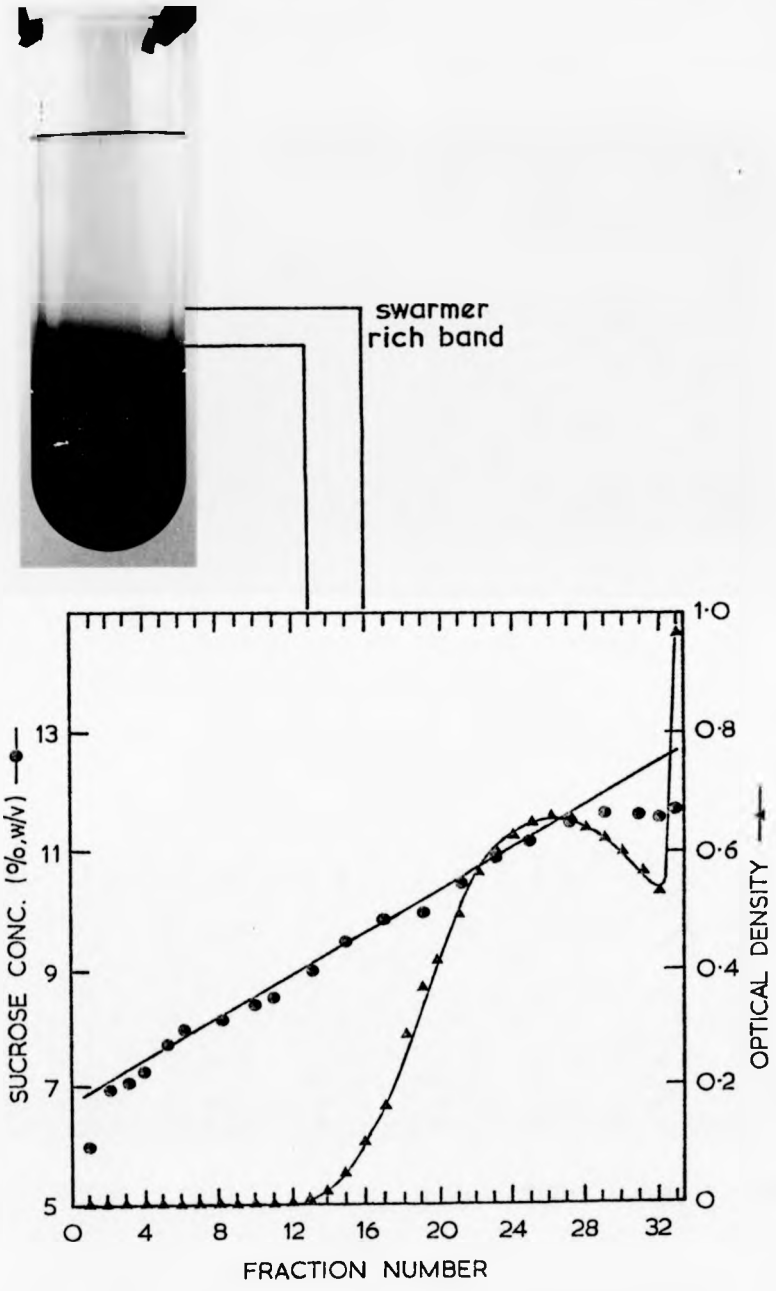

Fig. 51. Optical density distribution and approximate sucrose concentrations (estimated from refractivity) after centrifugation of sucrose gradient loaded with *R. palustris* cells.

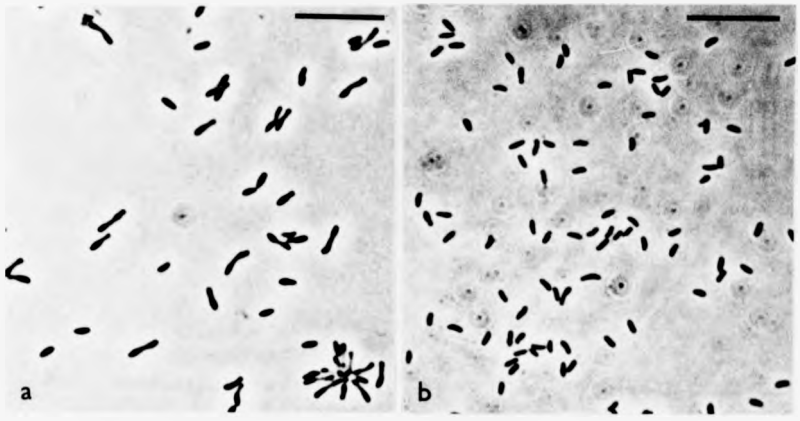

Fig. 52. Phase contrast photomicrographs of heterogeneous (a) and synchronous swarmer (b) populations of R. palustris. Bar represents 10 µm.

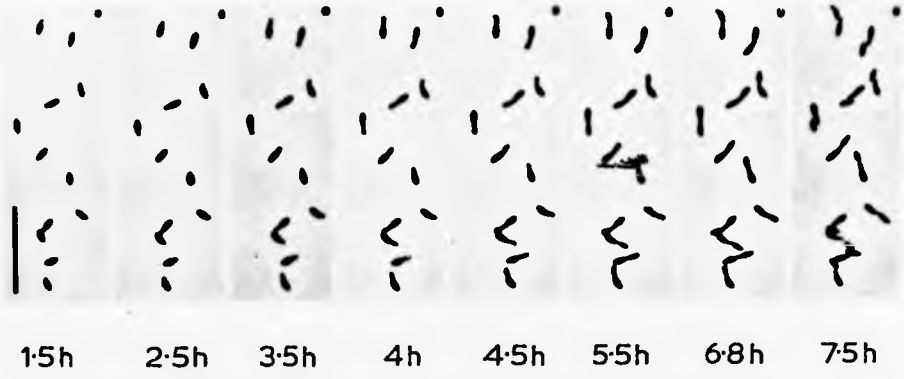

Fig. 53. Phase contrast photomicrographs of a synchronous R. palustris slide culture. Bar represents 10 µm.

with such swarmers a very well defined synchronous polar growth, budding and division was observed (Fig. 53). Again, the developmental cycle described by Whittenbury & McLee (1967) was confirmed. When numbers of individual cells were periodically counted in a field of such a synchronous slide culture an exponential plot of numbers with time revealed a very sharp first generation doubling at about 7.25 h (Fig. 54). In the second generation the increase in cell numbers was clearly seen to be biphasic. This resulted from first generation swarmer cells having to synthesise tubes before producing daughter buds, whilst mother cells budded directly on their pre-formed tubes. When cell numbers in a liquid culture of swarmers were followed by a viable plate count method a similarly abrupt first generation doubling of numbers was observed; however, this usually occurred at 5.25 h (Fig. 55a). When this doubling time was compared with those in the slide culture (Fig. 54) it was found to be very similar to the second generation swarmer doubling time. In contrast, the first generation doubling time in the slide culture was about 1.75 h greater. Thus there was an initial lag in growth on the slide culture, perhaps as a result of physiological differences between liquid and agar media.

Unfortunately, the stepwise cell number plot obtained by the viable count method gave rise to a considerable scatter of points. Consequently, when viable numbers were followed through two generations (Fig. 55b) the presence of the second generation biphasic doubling was hardly discernable. A similar scatter of points was obtained when total cell numbers were estimated in a synchronous liquid culture using a counting chamber (Fig. 55c).

An examination of more than 400 cells in half-hourly samples of the synchronous liquid culture used for Fig. 55a revealed that there was a tight grouping of appearance and disappearance of swarmers, cells with tubes , budding cells and cells having a division constriction (Fig. 56). Initially, more than 95% of cells examined were swarmers

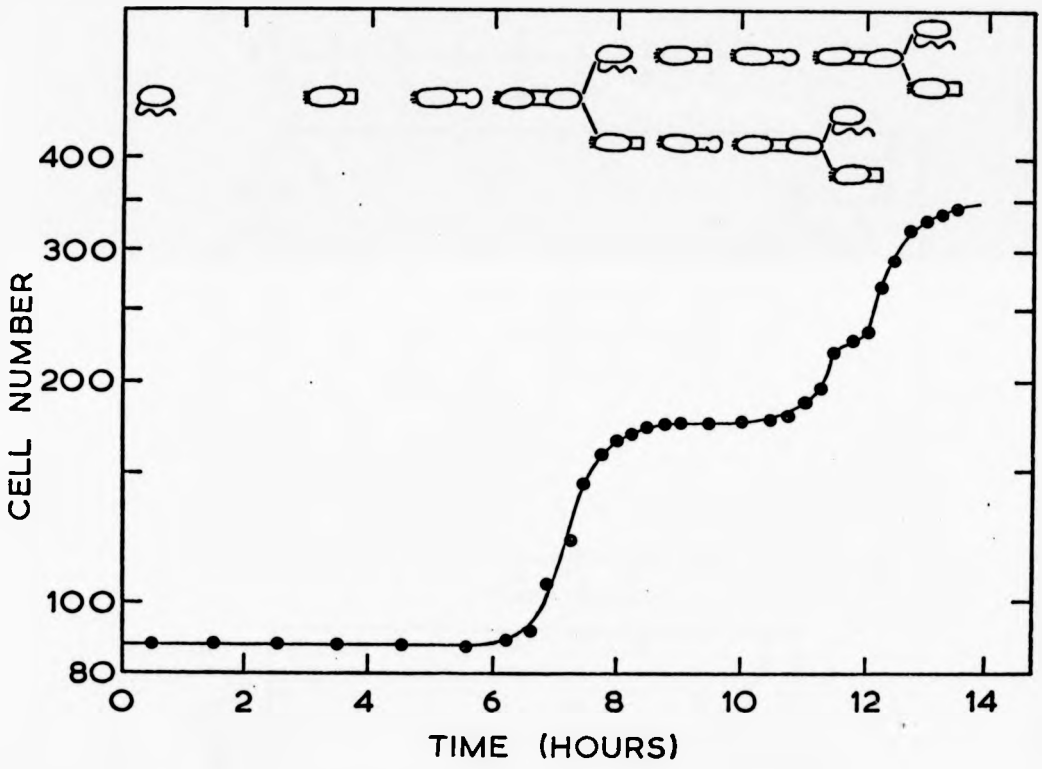

Fig. 54. Stepwise increase in cell numbers in a synchronous culture of R. palustris. The number of individual cells was followed in a field of a slide culture of swarmer cells.

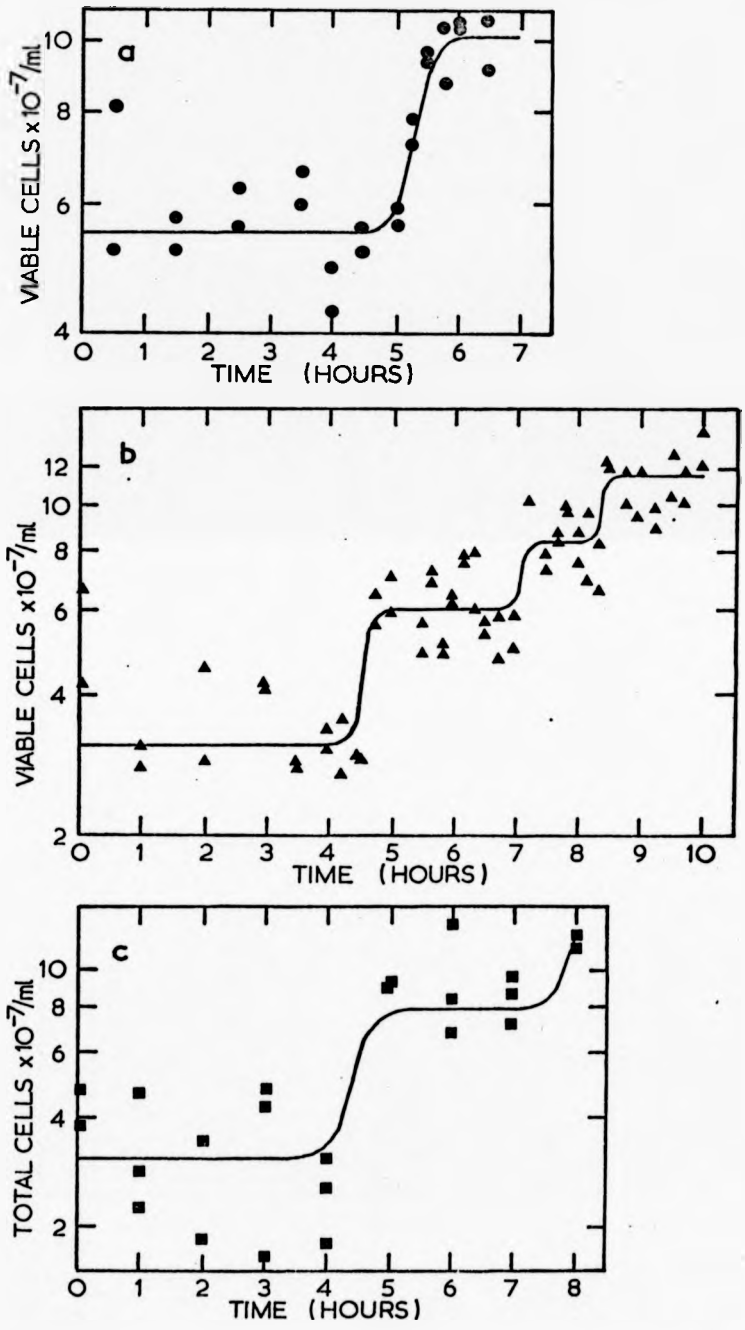

Fig. 55a-c. Cell counts of synchronous liquid cultures of R. palustris. Viable counts (a & b) were estimated from spread plates whilst total counts (c) were made using a bacterial counting chamber.

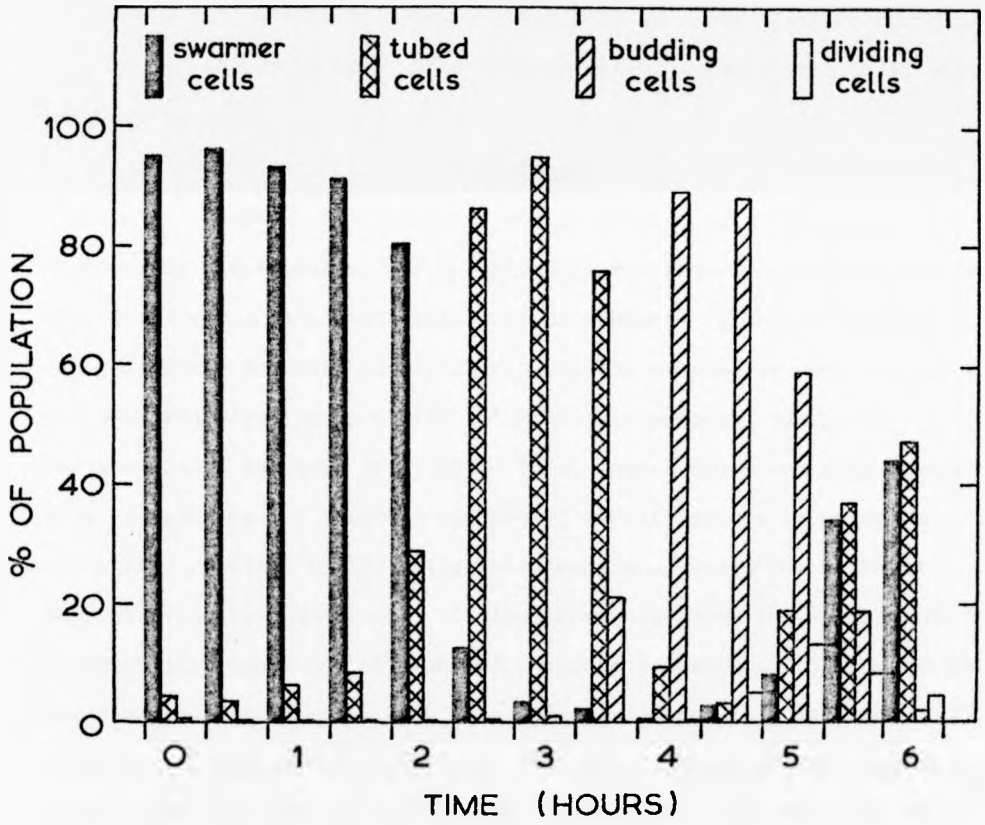

Fig. 56. Distribution of swarmer cells, cells with tubes, budding cells and dividing cells (those with a visible constriction) during synchronous liquid culture of *R. palustris*.

and remained as such for about 2 h. It was consistently observed that such a period of swarmer maturation occurred during development of R. palustris cultures. During bud formation there was still a peak of about 90% of examined cells all in the one stage of development. There was never a majority of cells having division constrictions observed since this stage in development was so short lived. Instead, the period of maximum cell division (Fig. 56) was seen as a new combined increase in swarmers and cells with tubes (mother cells) as well as a small peak of dividing cells.

Motility

A visual estimation of the proportion of motile cells in a synchronous liquid culture of R. palustris was made by examining sample cell suspensions in a bacterial counting chamber. Although the method did not permit accurate quantitative analysis it clearly demonstrated that the swarmers were highly motile but lost this property during the development of the tube (Fig. 57). Thus, some overlap was seen between loss of motility and holdfast synthesis, explaining the frequent observation of rapidly rotating motile cells which were stuck onto the slide or coverslip by their holdfasts. A close examination of motile cells in the electron microscope not only showed swarmers possessing flagella but also, occasionally, those cells with a holdfast and developing tube (Fig. 57). Motility had been almost completely lost by the stage of bud formation and the cells remained non-motile till just before cell division, when motile swarmers could be seen "towing" their non-motile mother cells behind them. After division the proportion of motile cells dropped, since half of the population were then non-motile mother cells.

Thus, in synchronous populations of R. palustris that were motile there was a defined cycle of motility with specific periods when flagella were synthesised and shed. Morphological aspects of development in synchronous cultures which were non-motile throughout the cycle

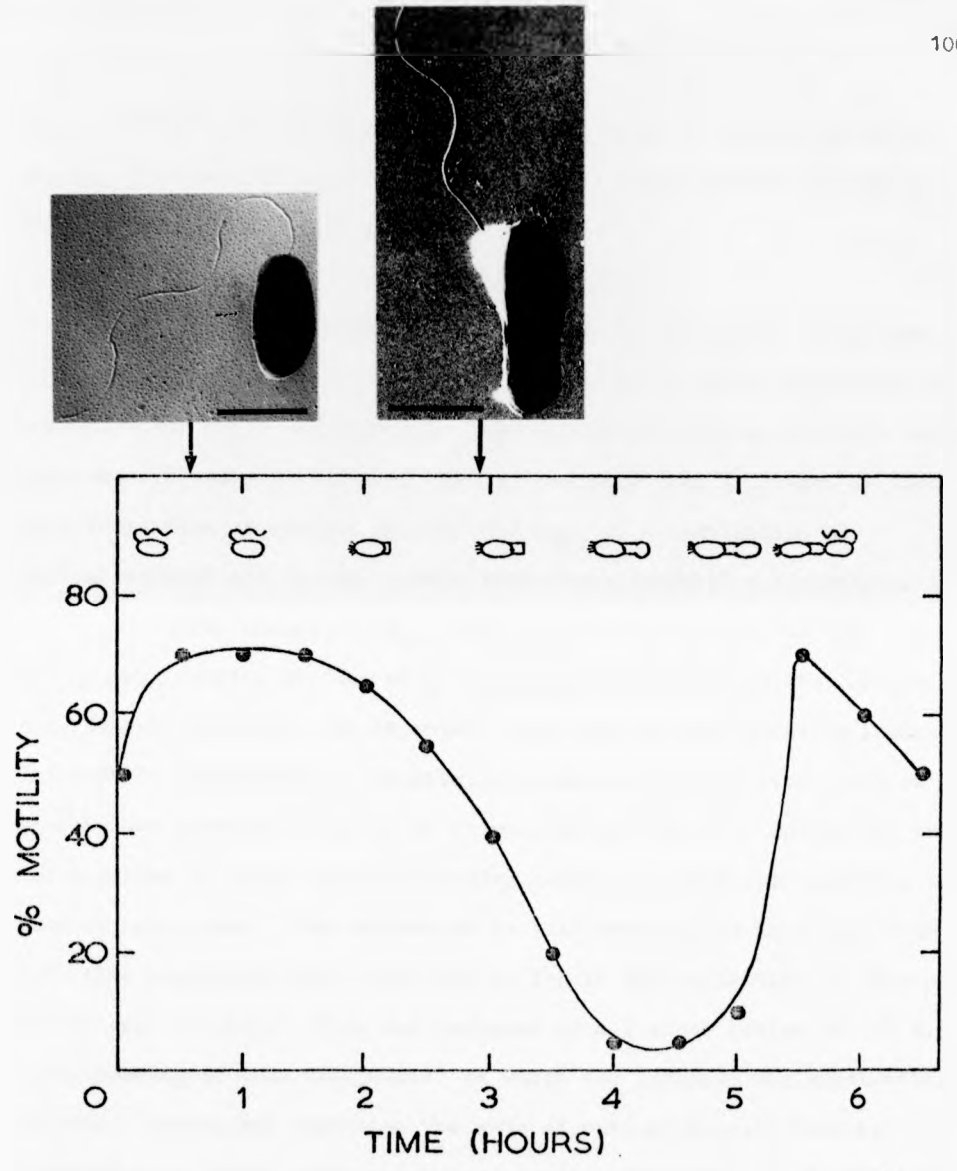

Fig. 57. Motility of R palustris during synchronous liquid culture. The proportion of motile cells was estimated visually at half-hourly intervals. The morphology of the synchronous culture is represented diagrammatically. Electronmicrographs were shadowed with gold-palladium alloy. Bars represent 1 μm.

(see Section I, p. 43) were precisely the same as in motile populations. Clearly the motility cycle is not an obligate or essential phenomenon in the development of R. palustris.

Optical density

It was initially intended that by following the extinction of synchronous cultures at 500 nm and 805 nm any periodic synthesis of photopigments could be monitored. Examination of spectra of whole cells suspended in media (Section I, Fig. 5) revealed that E_{500} was, in fact, only indicative of optical density and E_{805} of a combination of optical density and to some extent bacteriochlorophyll a adsorption.

When changes in E_{500} and E_{805} of a heterogeneous and synchronous cuvette culture of R. palustris were followed the results in Fig. 58 were obtained. As expected, there was an approximately logarithmic increase in extinction of the heterogeneous control; however, that of the synchronous culture increased in three distinct phases. Initially, there was a period of rapid increase lasting about 1.5 h and corresponding to swarmer maturation. The extinction at both wavelengths increased from zero time suggesting that there was no lag in the initiation of development in the swarmer cells. This was followed by a further period of 1.5 h, corresponding to tube elongation, in which the increase was substantially reduced. During bud formation the rate of optical density increase returned to a higher level which lasted until division. It is difficult to be certain precisely in what way the cellular development affected optical density. Whether the changes were due to altered refractility, opacity, cell size or shape, they do provide a useful, simple method of monitoring the progress of development in synchronous cuvette cultures.

Coulter counter analysis

The volume distribution obtained from a heterogeneous culture of R. palustris was typically positively skewed with a peak volume of about 0.38 μm (Fig. 59). This was probably due to the tendancy of cells

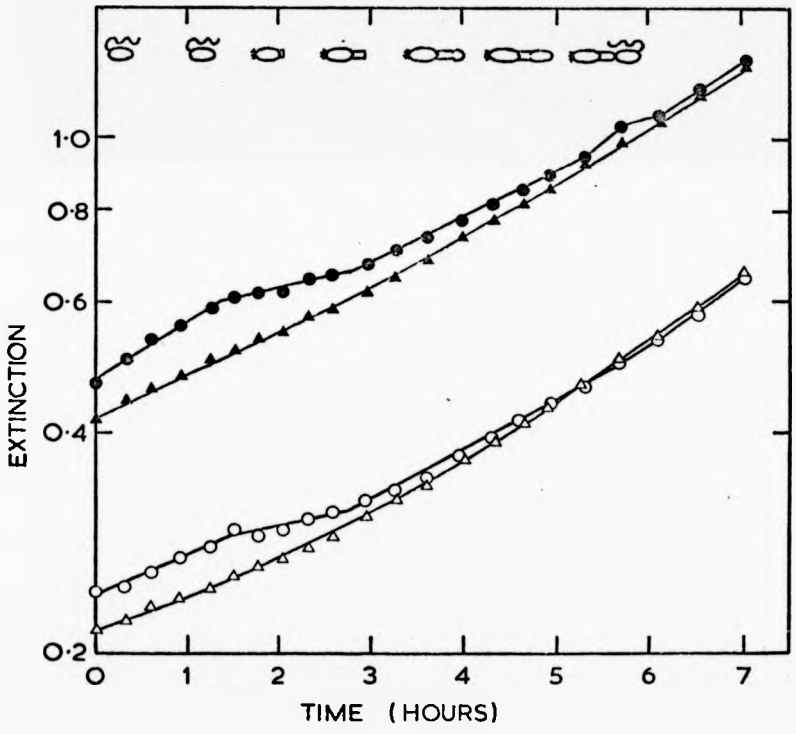

Fig. 58. Optical density changes in a synchronous culture of R. palustris. Extinction at 500 nm (closed symbols) and at 805 nm (open symbols) was compared in synchronous (○,●) and heterogeneous (△,▲) cuvette cultures.

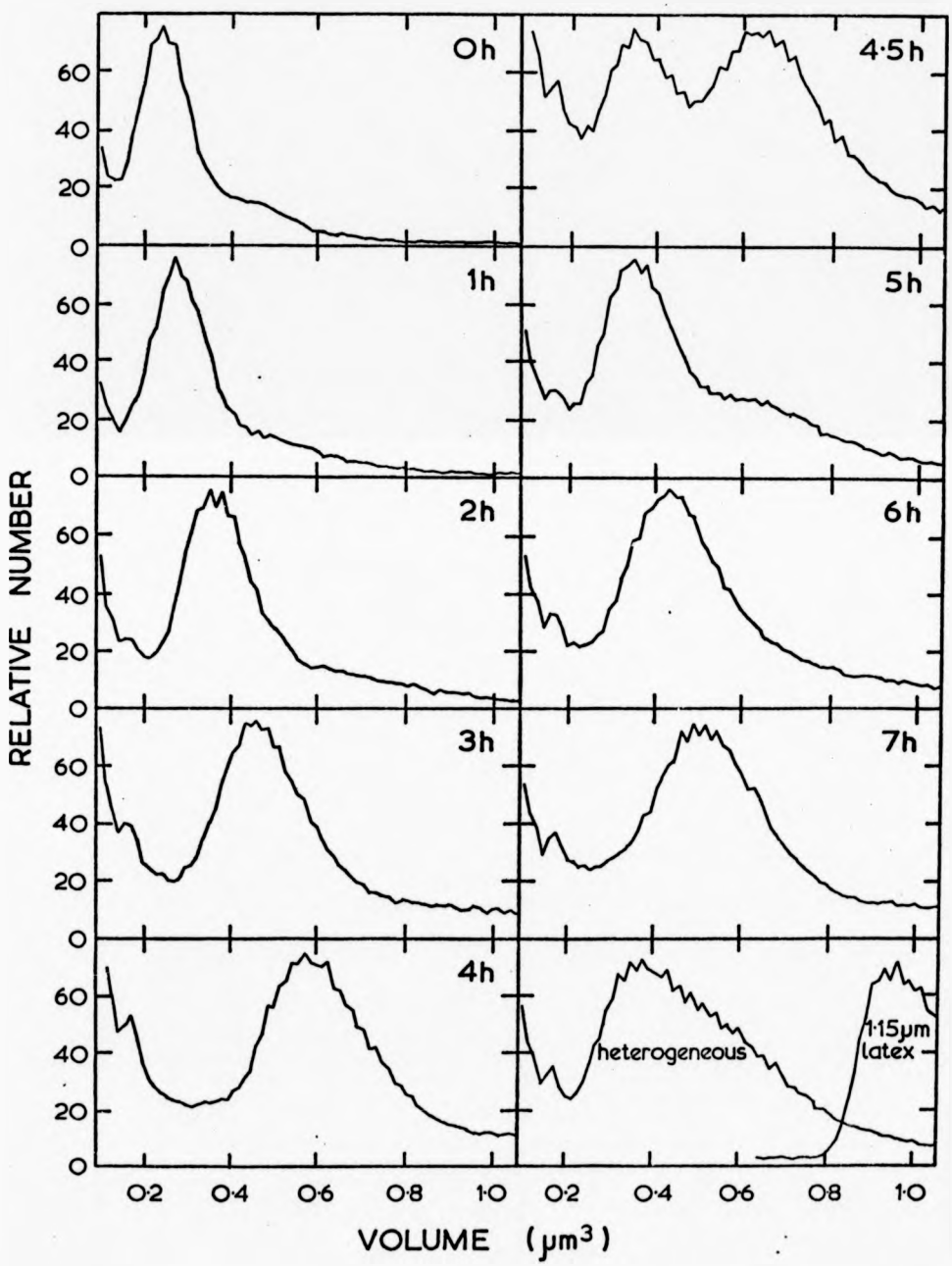

Fig. 59. Volume distributions of a synchronous culture of R. palustris. Samples were taken at intervals during development and volume distribution of suspended particles analysed using the Coulter counter and Channelyzer.

to form clumps and rosettes. When dilutions of samples from a synchronous liquid culture of swarmers were analysed on the Coulter counter volume distributions were initially very narrow, when compared with the heterogeneous culture, with a peak of about 0.24 μm^3 (Fig. 59). As development proceeded the peak volume increased until division (at about 4.5 h in this experiment) when two peaks were seen, one corresponding to a mixture of swarmers and mother cells (0.35 μm^3) and the other to cells about to divide (0.63 μm^3). When division was complete the resulting small volume cells again commenced their volume increase. These post-divisional cells had a mean volume about 45% greater than the original selected swarmers. This was possibly due in part to the selection of smaller than average swarmers on the sucrose gradient but more probably largely due to the presence of the larger mother cells together with the swarmers.

When changes in peak volume were plotted against a time scale (Fig. 60) it became clear that there was little volume increase during swarmer maturation; however the volume increase that followed this stage was linear.

Particle counts made on the synchronous culture sample dilutions (Fig. 60) again showed a very sharp increase in numbers at the time when cell division was observed. The doubling in numbers was not quite precise, presumably as a result of counting non-viable cells and non-bacterial particles.

The results presented here were obtained using cells which had lost their motility and were synthesising only small amounts of holdfast material. Motile populations were most unsuitable for analysis on the Coulter counter. Not only did small size debris, which may have been released holdfast material, interfere with the cell volume distribution plots, but in addition the doubling of particle numbers was almost totally obscured by clumping and rosette formation of cells.

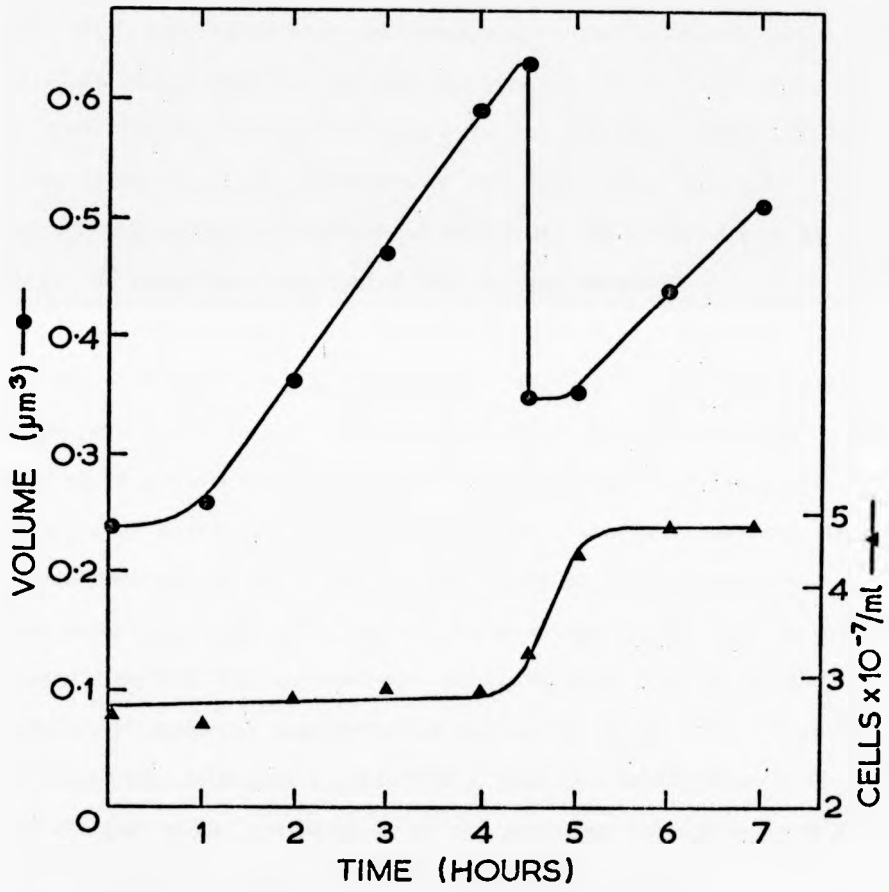

Fig. 60. Peak volume changes and total particle counts during a synchronous culture of R. palustris. The most frequent volumes observed in the distributions shown in Fig. 59 were plotted against time (●) and compared with particle counts (▲) obtained on analysis of the same samples with the Coulter counter.

Synthesis of nucleic acids

When samples of cells from a synchronous population were specifically stained for the location of nuclear material by the Giemsa stain method a pattern of genome replication could be vaguely discerned (Fig. 61) which correlated with that observed in thin sections (Section IIA, Fig. 33). It seemed that DNA replication was not commencing immediately but was most prominent over the last 3 h of the division cycle. Since the genome occupied a fair proportion of the cell volume and acid hydrolysis of RNA caused distortion of cells, it was difficult to be certain of the shape and location of the nuclear material.

In order to determine the timing of DNA synthesis more precisely the most convenient method would be incorporation of radioactive-labelled nucleotide precursors of DNA. By choosing suitable precursors RNA synthesis could also be followed. Unfortunately, adequate levels of incorporation of such nucleotides could not be achieved. However, some success was obtained by following incorporation of ^{32}P-phosphate into alkali-resistant material (DNA) whilst the remainder of the incorporation would largely be into RNA and membrane phospholipids. DNA synthesis in a synchronous culture was only detected during the final 2 to 3 h of the division cycle and coincided approximately with bud development (Fig. 62). The initial lag in DNA synthesis by synchronous populations was not due to physiological or nutritional shock since a heterogeneous culture, obtained by mixing all fractions from a sucrose gradient, incorporated ^{32}P into DNA approximately exponentially from 0 h (Fig. 62).

Incorporation of ^{32}P-phosphate into alkali-soluble fractions progressed in three stages (Fig. 62). During the first 1.75 h, corresponding to swarmer maturation, uptake was negligible and only when tube elongation started could incorporation be detected. At about 3.75 h, when bud development was beginning, there was an apparent increase in ^{32}P

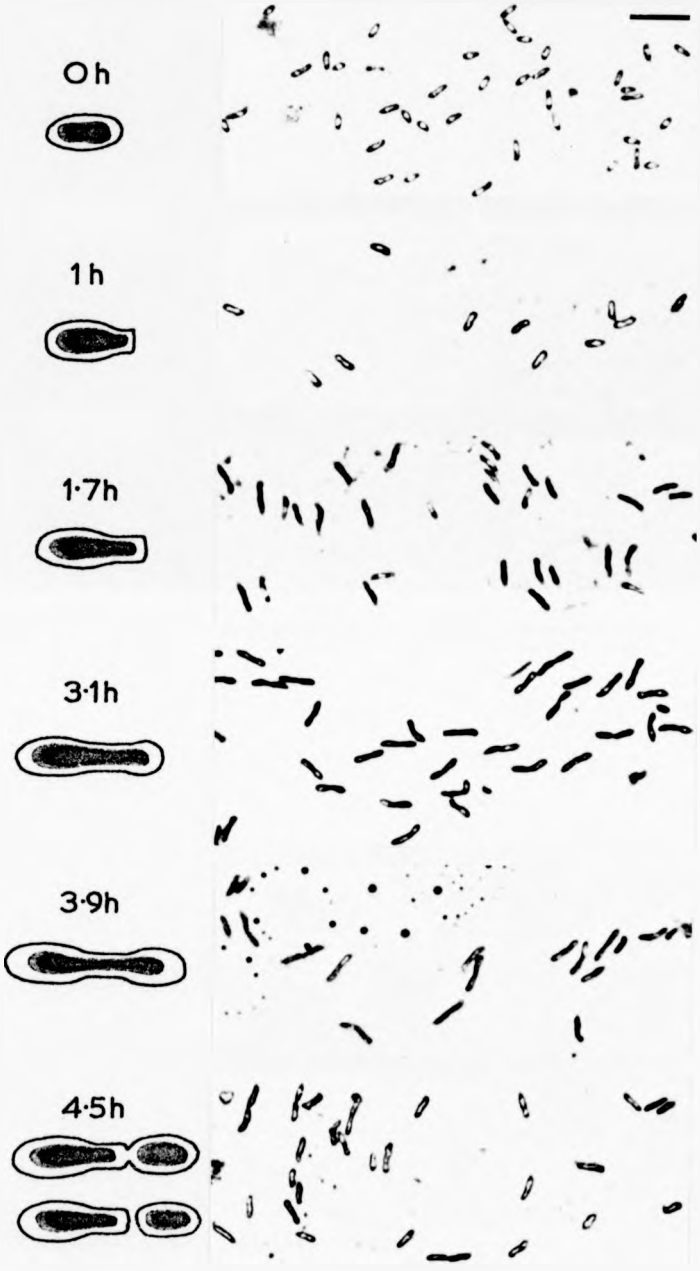

Fig. 61. Phase contrast photomicrographs of Giemsa-stained preparations of samples from a synchronous R. palustris culture. Bar represents 5 μm.

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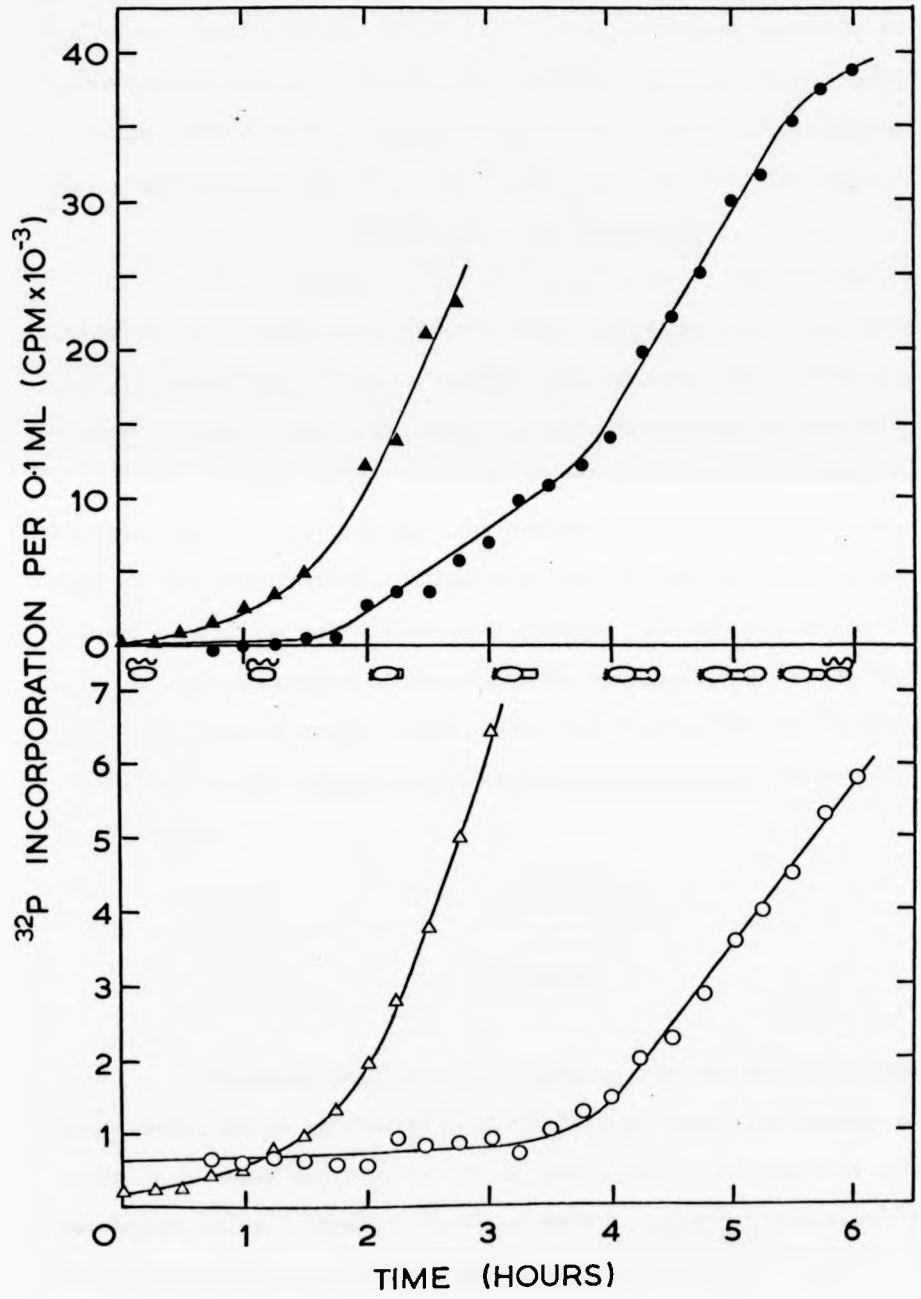
Fig. 62. Incorporation of ^{32}P-phosphate by R. palustris. Incorporation by a synchronous culture into alkali-soluble (●) and alkali-resistant (DNA,○) fractions was compared with incorporation into alkali-soluble (▲) and alkali-resistant (DNA,△) fractions of a heterogeneous control culture. Alkali-resistant incorporation points indicate the mean of duplicates.


40
30
20
10
0
7
6
5
4
3
2
1
0
1
2
3
4
5
6
^{32}P INCORPORATION PER 0·1 ML (CPM x10^{-3})
TIME (HOURS)

incorporation into alkali-soluble material which lasted until cell division. Again, uptake of ^{32}P into alkali-soluble material by a heterogeneous culture, prepared by mixing all fractions from a gradient, increased from 0 h (Fig. 62) showing that the lag in incorporation in the synchronous culture was a real feature of the division cycle.

Incorporation of ^{3}H-leucine

Incorporation of ^{3}H-leucine was used to monitor gross protein synthesis in a synchronous culture of R. palustris (Fig. 63). There was a rather slow initial rate of uptake when compared with a heterogeneous control culture. Even more noticable was a levelling and then drop of counts at, and just after, division of the synchronous culture whilst incorporation in the heterogeneous control continued. Thus it would seem that at division there was actually a loss of radioactive material from the cells into the culture medium. Whether this was concerned with synthesis of extracellular protein (such as flagellin) or with the actual division of cells is not known, but this pattern of ^{3}H-leucine incorporation was consistently observed in synchronous cultures of R. palustris.

DISCUSSION

Bacteria that divide by budding from the end of an appendage lend themselves to synchronisation by physical selection methods since recently released daughter cells are considerably smaller than all other developing cells. Thus it was found that R. palustris cells, collected from the uppermost bands in sucrose gradients, provided very homogeneous populations of swarmer cells. Similarly, Poindexter (1964) achieved synchronisation of the stalk-forming bacterium, Caulobacter, by centrifugation

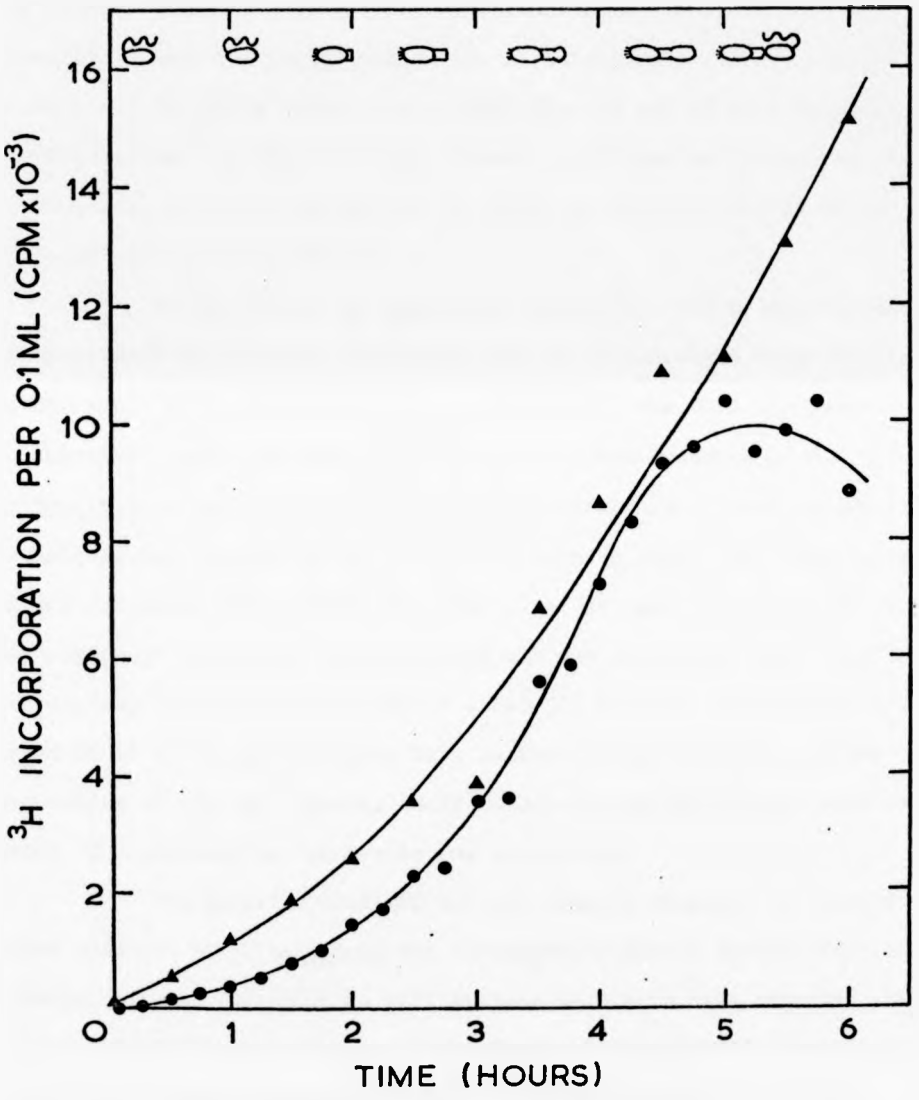


Fig. 63. Incorporation of ^{3}H-leucine by R. palustris. Uptake of ^{3}H-leucine was followed in a synchronous culture (●) and compared with that of a heterogeneous control (▲). Points indicate the means of duplicates.

but in sharp contrast to R. palustris the stalked cells had a lower sedimentation velocity than the swarmers and were therefore selected in the uppermost layers during centrifugation. This phenomenon has led to speculation that the Caulobacter stalk might naturally act as a floatation organelle. Tan et al (1974) found that only one out of five myxobacteria survived or was suitable for study after centrifugation in sucrose gradients. Fortunately, R. palustris appeared to be in no way affected by being suspended in sucrose gradients.

In synchronous R. palustris populations the appearance and disappearance of different cell types was closely grouped (Fig. 56) and maintained a high degree of synchrony throughout the division cycle. In contrast, Moore & Hirsch (1973a) reported that cultures of a Hyphomicrobium sp., synchronised by their centrifugation and filtration technique, had classes of different cell types present over broad spans of the estimated 13.5 h doubling time. Thus at zero time about 6% of the "synchronous" population had filaments and the proportion increased immediately, rising to a maximum of about 90% at 9 h. Cells with buds appeared at 2.5 h and increased to a maximum of just over 60% of the population at 10.5 h. Clearly their results revealed an unsatisfactory level of asynchrony in the developing population.

The stepwise doubling in cell numbers observed in swarmer slide cultures of R. palustris was considerably better defined than in a number of other synchronous cell systems that have been reported (Stove & Stanier, 1962; Moore & Hirsch, 1973a; Staley & Jordan, 1973; Tan et al, 1974). Perhaps the rather gradual cell number increases obtained by Stove & Stanier (1962) and Staley & Jordan (1973) with Caulobacter were largely as a result of rosette formation masking the true synchronous cell number doubling. The lack of precision in the cell number plots obtained from synchronous liquid cultures of R. palustris by total count of samples

using a counting chamber and by viable count methods (Fig. 55) was certainly in part due to the formation of rosettes by cells and the sticking of cells onto surfaces by means of their holdfasts. The inherent inaccuracy of the two methods would also have contributed to the scatter of points. Although the slide culture method of analysing stepwise doublings of synchronous cultures only gave numbers of relatively few cells, the members of the population examined were kept separated and this overcame any difficulties resulting from rosette formation and clumping. The method was extremely sensitive to cell number changes such as in the two-step second generation of synchronous cultures (Fig. 54). The 2-phase second generation and subsequent 4-phase third generation, 8-phase fourth generation, etc., leads to a far more rapid desynchronisation of swarmer cultures than is usual in synchronous cultures. Very complex patterns of biochemical and structural events can be predicted in the second and subsequent generations of such cultures. Similar problems are faced when studying synchronous cultures of any organisms which divide to give rise to two cells with different generation times. Presumably for this reason Moore & Hirsch (1973a,b) followed development of Hyphomicrobium only through the first generation. Degnen & Newton (1972a) were able to follow synchronous cultures of Caulobacter through to the second generation showing a biphasic doubling of particle counts. However, they did not follow rates of DNA synthesis into the second generation. Staley & Jordan (1973), who followed a synchronous Caulobacter culture through three supposed synchronous doublings, incorrectly assumed single-phase cell number increases to occur through the second and third generations, rather than 2-phase and 4-phase doublings. In actual fact, after the initial lag of 1.5 h cell number increases that they showed were almost linear for the next 4.5 h. Bearing these problems in mind, R. palustris was primarily studied only through the first generation. This does not lend

much weight to the reproducibility of patterns observed in the first swarmer cell cycle to those in the second swarmer cycle in a synchronous culture. However, such information is difficult to obtain where the second generation population is of two classes of cells.

Since R. palustris possesses sticky holdfasts on all cells except swarmers it is possible that selection of swarmers may be achieved from mother cells adhered to solid surfaces. The membrane technique of Helmstetter & Cummings (1963,1964) and glass bead methods such as that of Brandon & Norris (1972) might both be useful in this respect especially if a continuous supply of swarmers was required. Alternatively, larger numbers of swarmers might be obtained by continuous-flow size selection using a zonal rotor (Lloyd et al, 1975). Nevertheless, sufficient swarmers were obtained by sucrose gradient centrifugation to allow embedding and sectioning of cells (Section IIA) as well as various biochemical and structural changes with time to be examined.

The observation of a cycle of motility and non-motility in R. palustris is by no means unique. It is interesting to note that all appendaged bacteria that have been described are motile at certain stages of their developmental cycles. Leifson (1964), in his initial description of H. neptunium, demonstrated a cycle of motility by staining flagella. This cycle was identical to that observed here in R. palustris. Shapiro & Maizel (1973) have investigated the motility cycle of C. crescentus. They used the fact that intact flagella were released from developing swarmers into the medium to enable them to examine the structure of the flagella. They also showed that new flagella were synthesised on developing swarmer cells 30 to 40 min before cell division. Dow (1974) observed massive aggregates of flagella by light microscopy of Rm. vanniellii. This was as a result of the gross shedding of peritrichous flagella from developing swarmer cells. Similarly, he was able to use the shedding

phenomenon to permit easy isolation and purification of flagellin. Clearly a considerable degree of cellular control is involved, not only in inducing the release of flagella at specific times, but also in synthesising flagella at specific new locations, at specific times and at very rapid rates of synthesis.

The cuvette culture technique provided a convenient method of accurately following continuous optical density changes in a synchronous culture. The Suba-Seal in the top of the cuvette allowed occasional small samples to be removed for morphological examination. Although the anaerobic cuvette described by Hodgson, McCord & Fridovich (1973) permitted bubbling of gasses through the liquid, sampling of the contents could not be achieved. The pattern of optical density increase in synchronous cultures showed that development started from 0 h and that there was no lag caused by the synchronisation procedure. The characteristic three-stage optical density increase which was observed has similarly been demonstrated in synchronous cultures of Rm. vannielii swarmers (Dow, 1974). The three phases in Rm. vannielii coincided with swarmer maturation, filament outgrowth and bud development in much the same way as in R. palustris. During filament and tube outgrowth the cellular extentions of Rm. vannielii and R. palustris, respectively, develop with no internal photosynthetic membranes. One explaination of the optical density phenomenon, therefore, is that such parts of cells have quite different optical properties, perhaps scattering less light and resulting in apparently lower rates of optical density increase during their synthesis.

Few other workers have followed the changes of cell volume during the course of synchronous development of cells. Experiments have most commonly been carried out comparing volume distributions in populations of cells harvested from different levels in density gradients (Kubitschek, Bendigkeit & Loken, 1967; Manor & Haselkorn, 1967). Clark (1968) followed

cell volume distributions in synchronous cultures of E. coli B/r and found a pattern of volume increase identical to that observed in R. palustris with the exception that the initial lag corresponding to R. palustris swarmer maturation was absent. Dow & Whittenbury (in preparation) have followed volume changes in synchronous cultures of Rm. vannielii. The most significant departure from the pattern observed in R. palustris was that no gradual shift in volume distribution was observed during filament formation and early bud formation. Instead, there was a later change in apparent volume, through to a double peak, and then on to form a single large volume peak. Since Rm. vannielii does not divide, but cells become independent by plug formation (Murray & Douglas, 1950) the large volume peak remained. Presumably, therefore, the Coulter counter did not detect the filament and only recognised the mother cell and mature daughter cell connected by a filament as a single large impulse.

As will later be seen in Section IIC of this thesis the incorporation of ^{32}P-phosphate into alkali-resistant fractions is almost completely inhibited by nalidixic acid. Since this antibiotic is generally regarded as being a specific inhibitor of DNA replication the method used here of following ^{32}P incorporation into alkali-resistant material is indicative of DNA synthesis. DNA replication in Hyphomicrobium strain B-522 has been reported to occur during swarmer maturation, hyphal development and the beginning of bud formation (Moore & Hirsch, 1973b). It has also been found that DNA replication in Rm. vannielii occurs during hyphal elongation (Dow, 1974) and that nuclear bodies separate before bud development (Murray & Douglas, 1950). Therefore the pattern of DNA replication in R. palustris is quite different from previously examined filament-forming, budding bacteria. It has already been mentioned that Caulobacter, although not a budding organism, resembles R. palustris in many ways. Degnen & Newton (1972a) demonstrated a pre-synthetic gap in the pattern of

chromosome replication in Caulobacter, similar to that seen here in R. palustris, as well as a post-synthetic gap before cell division. In both R. palustris and Caulobacter cellular development occurs for some time before the process of cell duplication commences. It is possible that the late DNA replication is adequate in these organisms as there is no restriction presented to the location of the DNA at the division plane and this division plane does not become established until the preliminary development is complete. Hyphomicrobium and Rhodomicrobium form narrow filaments which would not allow positioning of the chromosome at the division plane prior to cell separation or plugging. Consequently DNA replication and segregation occur earlier in the cell cycles. Various aspects of the timing of DNA replication and cell division will be considered in much more detail later in this thesis.

^{32}P incorporation into alkali-soluble material may not solely have represented RNA synthesis. Phospholipid phosphate would also be alkali-soluble. Since photosynthetic membranes are not observed in the tube of cells it is unlikely that the alkali-soluble incorporation immediately after swarmer maturation was, to any great extent, into membrane phospholipid. This incorporation was probably largely into RNA. It was only after bud formation that large quantities of phospholipid would be synthesised and might account in part for the increased rate of incorporation into alkali-soluble material, in spite of the fact that much of this incorporation would be solublised by washing with ethanol and ether.

Incorporation of ^{3}H-leucine was certainly indicative of protein synthesis and this protein synthesis was noticably discontinuous, especially during cell division. Reviewers tend to summarise that RNA and protein synthesis are continuous in the cell cycle of bacteria (Halvorson et al, 1971); however budding bacteria cannot necessarily be considered in these generalisations.

A number of points came to light when the division cycle of R. palustris was considered as a combination of the various morphological, ultrastructural and biochemical changes. A phase of maturation of swarmers was seen, usually occupying 30 to 35% of the cell cycle. This compares closely with the proportion of the division cycle spent in swarmer maturation in Hyphomicrobium (Moore & Hirsch, 1973a) and in Rm. vannielii (Low, 1974). R. palustris swarmer maturation was characterised by the rapid increase in optical density, the slight delay in volume increase and the lack of apparent morphological development, DNA synthesis and RNA synthesis. These features and the ensuing holdfast synthesis, RNA synthesis, tube formation and loss of flagella are all phenomena unique to the developing daughter cells. Once a daughter cell has carried out these developmental steps it becomes, and remains, a mother cell which will continue to give rise to successive daughter buds, apparently for an indefinite period of time. It would seem, therefore, that certain genes of the R. palustris chromosome are only expressed during swarmer maturation and tube elongation and then never again. It is also essential that immediately after separation of replicated nuclear material differential control of the two gene copies must occur, such that one will continue to synthesise new daughter cells and the other will code for swarmer maturation and tube elongation.

Another important factor in the development of budding and prosthecate bacteria is the localisation of cellular growth and positional development of organelles. Cellular growth is apparently localised at one pole of the cell, whilst holdfast synthesis, if it does occur, is always at the opposite pole (Leifson, 1964; Whittenbury & McLee, 1967; Pfennig, 1969). It has been reported that the extreme tip of the developing daughter cell of Hyphomicrobium strain B-522 usually becomes the point of hyphal synthesis after cell division (Moore & Hirsch, 1973a). In R. palustris

the direction of growth was reversed in the daughter cell, tube elongation occurring at the site of cellular division. On slide culture, therefore, microcolonies formed by cells stacking side by side as mother and daughter cells elongated towards each other. What defined the site at which cell elongation would occur, relative to other cell components, is not known.

Clearly the developmental cycle of R. palustris presents a model suitable for studying differentiation in which a wide variety of temporal and positional controls must be exerted, in spite of the comparative simplicity of the cycle. The nature of such controls can best be established if some knowledge is obtained of the effect of modifying the controls. This might be achieved by blocking positive or negative controls by treatment with inhibitors or stimulators or by obtaining suitable mutants.

SECTION II: STUDIES ON THE CELL CYCLES OF SOME BUDDING RHODOSPIRILLACEAE

C: EFFECTS OF ANTIBIOTICS ON THE CELL CYCLE OF R. PALUSTRIS

SECTION IIC: EFFECTS OF ANTIBIOTICS ON THE CELL CYCLE OF R. PALUSTRIS

INTRODUCTION

A large number of antibiotics are now available which exhibit well established and specific inhibitory actions (Gale *et al*, 1972) and such compounds offer an invaluable tool for studying differentiation and morphogenesis. By studying the development of cells after treatment with antibiotics specific for particular events in the cell cycle it is possible that the interdependence of such events could be determined. Events observed in the cell cycle are not necessarily essential: it has already been shown that motility of *R. palustris* is not a prerequisite of holdfast synthesis or normal ordered development within the cell cycle.

De novo membrane synthesis in the daughter bud of a developing *R. palustris* cell and the concommitant bacteriochlorophyll synthesis are particularly interesting features of the developmental cycle. It is not known whether bud development can continue if bacteriochlorophyll synthesis is inhibited. Amitrol (3-amino-1,2,4-triazole) is known to be a fairly specific inhibitor of carotenoid and chlorophyll synthesis in plants and algae (Kirk & Tilney-Bassett, 1967), although it is not clear whether it would have a similar action on photosynthetic bacteria. Application of this compound to heterogeneous cultures of *R. palustris* might reveal inhibition of growth due to lack of pigment synthesis, in which case it could possibly be used in studying the dependence of daughter development on photopigment synthesis.

When synthesis of DNA is specifically inhibited in certain Gram negative bacteria cell division is seen to be inhibited, but only after a delay. Thus Helmstetter & Pierucci (1968) demonstrated the inhibition of cell division in *E. coli* 20 min subsequent to inhibition of DNA replication by u.v. irradiation or treatment with nalidixic acid (NAL)

or mitomycin C. It is now well established that there is a period after completion of chromosome replication in E. coli when cell division is insensitive to the effects of such DNA synthesis inhibitors (Clark, 1968; Dix & Helmstetter, 1973; Burdett & Murray, 1974). Similarly, studies on the effects of mitomycin C and hydroxyurea on DNA synthesis and the continuation of cell division in C. crescentus have indicated a coupling of cell division to completion of chromosome replication (Degnen & Newton, 1972b). Weiner & Blackman (1973) found that NAL specifically inhibited bud formation as well as DNA replication in H. neptunium and later showed that the bud formation and separation could be inhibited by NAL during slide culture of the organism (Blackman & Weiner, 1975). In complete contrast, however, it has been shown that after DNA synthesis was inhibited by treatment of B. subtilis with NAL or by thymidine starvation of thy⁻ mutants, cell division continued for a considerable period of time giving rise to enucleate cells (Donachie, Martin & Begg, 1971). Clearly cell division was not coupled to chromosome replication in this organism.

It is possible, therefore, that DNA replication in R. palustris may be linked to other events in the development cycle such as bud formation or cell division. It must first be established whether development is arrested at any particular stage and what other events occur after inhibition of DNA replication. As has been outlined above, several inhibitors of DNA replication have been used by other workers. Mitomycin C and NAL have probably been used most frequently. The mode of action of mitomycin C is quite well understood, inhibition being a result of covalent cross-linking of mitomycin molecules between complementary strands of DNA (Szybalski & Iyer, 1967). As a consequence the inhibitory action is irreversible. In addition massive degradation of pre-existing DNA is observed although synthesis of RNA and protein is unaffected (Reich, Shatken & Tatum, 1961). NAL is a preferential inhibitor of DNA replication

in both Gram negative and Gram positive bacteria (Goss, Deitz & Cook, 1964, 1965; Cook et al, 1966). Unfortunately, there has been little success in elucidating its mode of action (Boyle, Cook & Goss, 1969; Bourguignon, Levitt & Sternglanz, 1973), but it does have the advantage that the inhibitory action of NAL is reversible (Ward, Hane & Glaser, 1970) and little degradation of pre-existing DNA occurs. As a result of the latter two points, studies on the effect of inhibiting DNA replication in R. palustris were mainly achieved by the use of NAL.

The specific action of penicillin in weakening the cell wall at the point of wall growth has been used by several workers to demonstrate the site of new wall growth in bacterial cells (Schmidt & Stanier, 1966; Schwartz et al, 1969; Donachie & Begg, 1970). Although the precise molecular basis for the action of penicillin is not clear (Gale et al, 1972) its specificity in inhibiting bacterial cell wall synthesis has been known for some time (Duguid, 1946; Park, 1952; Park & Strominger, 1957). It has become well established that penicillin can inhibit the transpeptidase and carboxypeptidase reactions in cross-link formation between mucopeptide subunits of the cell wall thus weakening the newly synthesised wall structure (Tipper & Strominger, 1965; Park, 1966; Izaki, Matsuhashi & Strominger, 1968). More recently, Hartmann, Holtje & Schwartz (1972) have demonstrated that murein hydrolases, which cleave the cell wall murein to allow insertion of new mucopeptide for wall growth, are also directly inhibited by penicillin. Application of penicillin to R. palustris cultures might permit the demonstration of true polar growth of the organism as well as distinguishing whether tube elongation occurs by wall growth at the base or the tip of the tube.

Development in sporulating B. subtilis (Sterlini & Mandelstam, 1969). Myxococcus xanthus (Ramsey & Dworkin, 1970), Dictyostelium discoideum (Ashworth, 1971) and possibly C. crescentus is thought to involve

the synthesis of long-lived m-RNA and sequential regulation at the level of translation. Because of the synthesis of stable m-RNA it is possible for transcription of genes for a particular event to be detected a considerable time before translation commences. This gives rise to multiple points of commitment in the developmental sequence (Mandelstam, 1971). One method by which stable m-RNA can be recognised is by observing the appearance of a particular event when transcription or translation have been inhibited at different times during the developmental sequence. Thus treatment with rifampicin, which selectively inhibits DNA-dependent RNA polymerase (Hartmann et al, 1967), after long-lived m-RNA has been synthesised for an event would not inhibit completion of that event but would inhibit subsequent events. On the other hand, immediate inhibition of the event by chloramphenicol, which inhibits translation (Gale et al, 1971), would show that translation of m-RNA for that event was in progress and long-lived enzymes were not present. Since it is conceivable that such control mechanisms could occur in R. palustris a few preliminary experiments were also carried out to examine the effects of rifampicin and chloramphenicol.

MATERIALS AND METHODS

Cultivation and examination of cells. R. palustris C1 was used in all experiments and grown in PAYE medium (Section IIA, p. 61). A 1% inoculum was used for 100 ml heterogeneous liquid cultures in 250 ml flasks gassed with oxygen-free nitrogen (Section I, p. 30). Cultures were incubated at either 34°or 36° depending on the desired rate of growth and were illuminated at an intensity of 4,000 lux. Cultures were generally used after about 22 h incubation (mid-exponential phase of growth) when preparing synchronous cultures.

Synchronous populations were prepared by the modification of the differential sedimentation method of Mitchison & Vincent (1965) as described in Section IIB (p. 94). The analysis of volume distributions in synchronous cultures using the Coulter counter and Channelyzer were also described in Section IIB of this thesis (p. 96).

Slide cultures and microscopy of cell populations were carried out as described in Section IIA (p. 61) and the Giemsa staining as in Section IIB (p. 96). Gold-palladium shadowed preparations and ultrathin sections were prepared and examined in the electron microscope as in Section IIA (p. 62).

Antibiotics. The sodium salt of benzylpenicillin (Crystapen; Glaxo Laboratories Ltd, Greenford) was dissolved in PAYE to give a stock solution of 200,000 IU/ml and used at a final concentration of 4,000 IU/ml. Rifampicin (Sigma Chemical Co., London) was dissolved in PAYE by adding the weighed crystals, progressively over a period of 2 to 3 h, into the measured stirred liquid maintained at 4° in a cold room. Stock solutions at 2 mg/ml were stored at -20° but only thawed once for use. Cultures were treated with a final concentration of 100 µg/ml rifampicin. Amitrol (3-amino-1,2,4-triazole), chloramphenicol and nalidixic acid (NAL) were all obtained from Sigma, prepared as aqueous solutions and used at a concentration of 50 µg/ml. Stock aqueous solutions of NAL were prepared by dispersing 1 mg/ml antibiotic in PAYE or distilled water and dissolved by addition of NaOH to a concentration of 7 mM. No change in pH of culture media was observed on addition of stock solutions of NAL. Aqueous stock solutions of mitomycin C (Sigma) were prepared in multidose vials containing 2 mg mitomycin C and 48 mg NaCl to increase solubility and were used at a final concentration of 10 µg antibiotic/ml.

If required, all the above antibiotics were sterilised by filtration through Millipore 0.22 µm GS filters.

Incorporation of radiochemicals. Incorporation of ^{32}P-phosphate into alkali-soluble and alkali-resistant fractions was estimated by the method described in section IIB (p. 97). ^{32}P-phosphate was added to give an activity of 6.25 µCi/ml in the culture medium, which consisted of PAYE containing only 1 mM phosphate buffer.

^{3}H-leucine incorporation was measured as described in Section IIB (p. 97) in samples of cultures grown in the presence of 5 µCi/ml ^{3}H-leucine, 10 µg/ml unlabelled leucine and PAYE medium containing only 0.05 g/l yeast extract.

RESULTS

Penicillin-induced spheroplast formation

When a heterogeneous population of R. palustris was grown by slide culture at 34° in the presence of 4,000 IU/ml penicillin and 15% sucrose as an osmotic stabiliser, spheroplasts were seen to emerge from the cells within 1 to 1.5 h (Fig. 64). Close examination of the populations revealed that spheroplasts were always formed at one end of the cells regardless of the stage of development. This clearly demonstrated the polar nature of cell wall growth. It is especially important to note that it indicated that wall growth of the tube was from the tip and not the base. Unfortunately, dividing cells were not seen with spheroplasts present, but it would be anticipated that during the course of constriction wall growth would be occurring at the division plane allowing this new growth region to become the site of growth of cells after division.

Effects of NAL, rifampicin, chloramphenicol and amitrol on growth

Heterogeneous cuvette cultures were treated, after 2 h growth at 34°, with appropriate concentrations (see Materials and Methods) of NAL, rifampicin, chloramphenicol and amitrol. The extinction of the

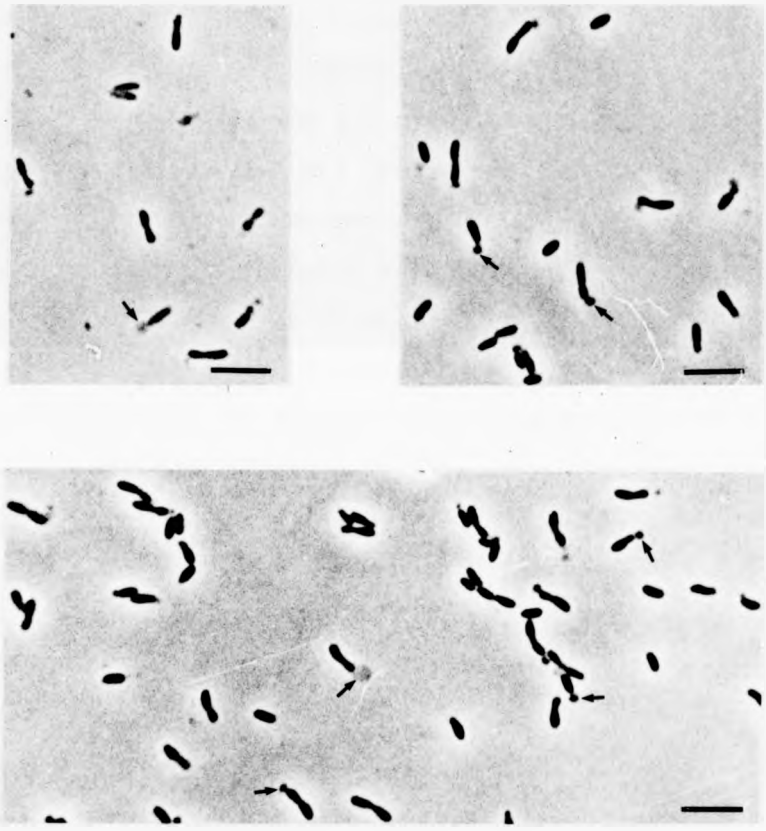


Fig. 64. Phase contrast light photomicrographs of penicillin-treated R. palustris cells. A heterogeneous population was grown by slide culture in the presence of 4,000 IU/ml penicillin and 15% sucrose and examined for spheroplasts (↑) after 1 to 1.5 h. Bar represents 5 µm.

cultures at 500 nm and 805 nm were followed before and after addition of antibiotics and compared with control cultures (Fig. 65). The extinction of rifampicin-treated cultures was only measured at 805 nm as the antibiotic absorbed light at 500 nm. Amitrol and NAL gave rise to only very slight changes in the optical density, suggesting that they had no inhibitory effect on cell growth. On the other hand, rifampicin and chloramphenicol treatment resulted in a substantial reduction in the rate of optical density increase which in fact ultimately ceased. Comparison of the deviation of optical densities in treated cultures from those in the controls (Fig. 66) showed that the inhibition by rifampicin did not manifest itself until 1 h after addition, whilst chloramphenicol inhibition was detected within 10 min. The delay in rifampicin inhibition suggests the involvement of stable m-RNA species in the growth of R. palustris.

Effects of rifampicin and chloramphenicol on ^{3}H-leucine incorporation

Some delays in the inhibitory actions of rifampicin and chloramphenicol in the previous experiment may have been as a result of protein synthesis inhibition not immediately becoming manifest in optical density changes and not due to stable m-RNA. In order to negate such delays the inhibition of incorporation of ^{3}H-leucine by treatment of heterogeneous populations with rifampicin or chloramphenicol was investigated (Fig. 67). Three parallel cultures grown at 34^{o} showed very similar patterns of incorporation during the first 3 h, at which time the antibiotics were added. Although the control culture continued to incorporate ^{3}H-leucine for the remaining 3 h of the experiment, chloramphenicol inhibited incorporation almost immediately. Chloramphenicol was therefore rapidly inhibiting protein synthesis as well as the subsequent optical density increases. On the other hand, incorporation by the rifampicin-treated culture stopped only after a delay. This resulted in a higher final level of incorporated ^{3}H-leucine than was observed in the

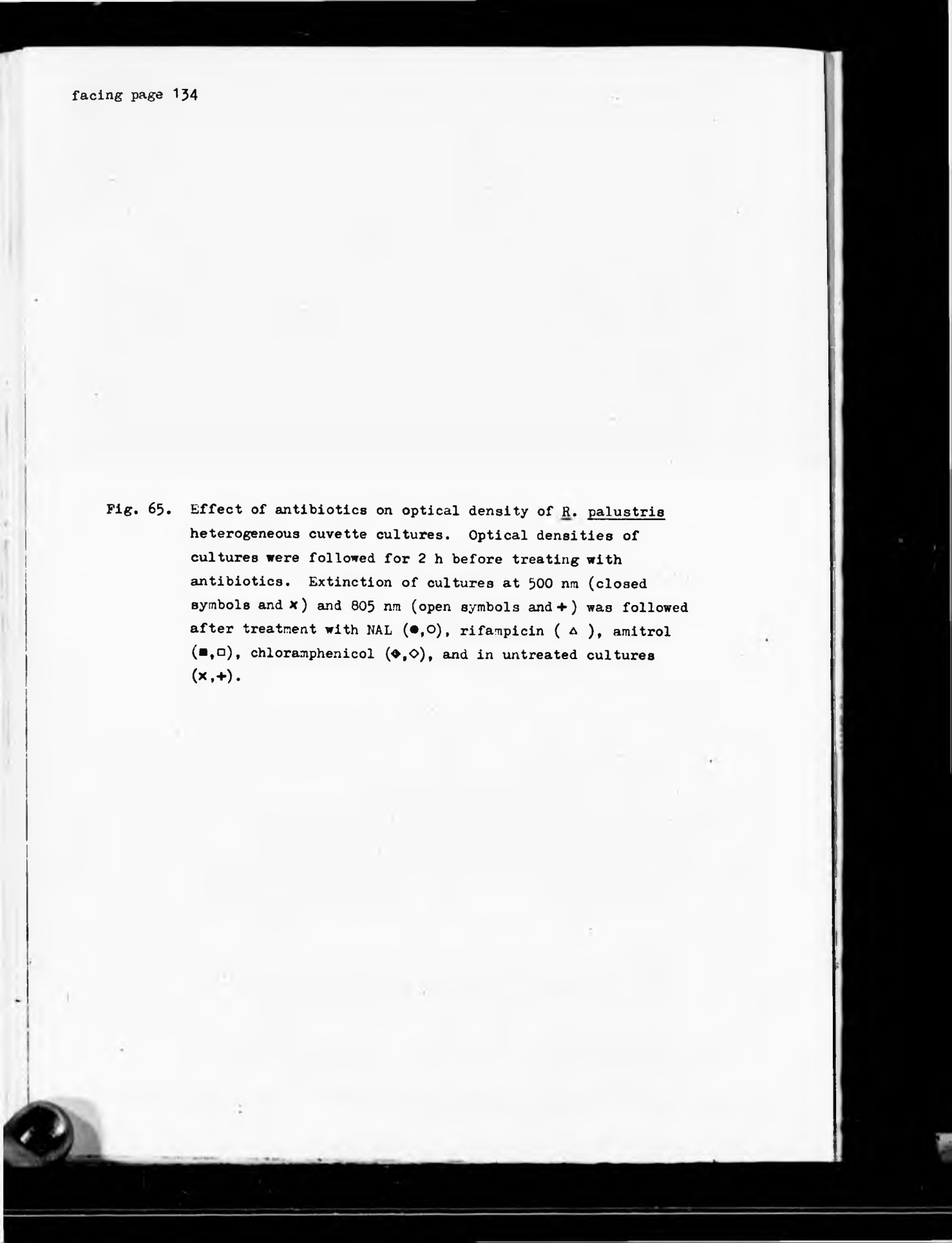
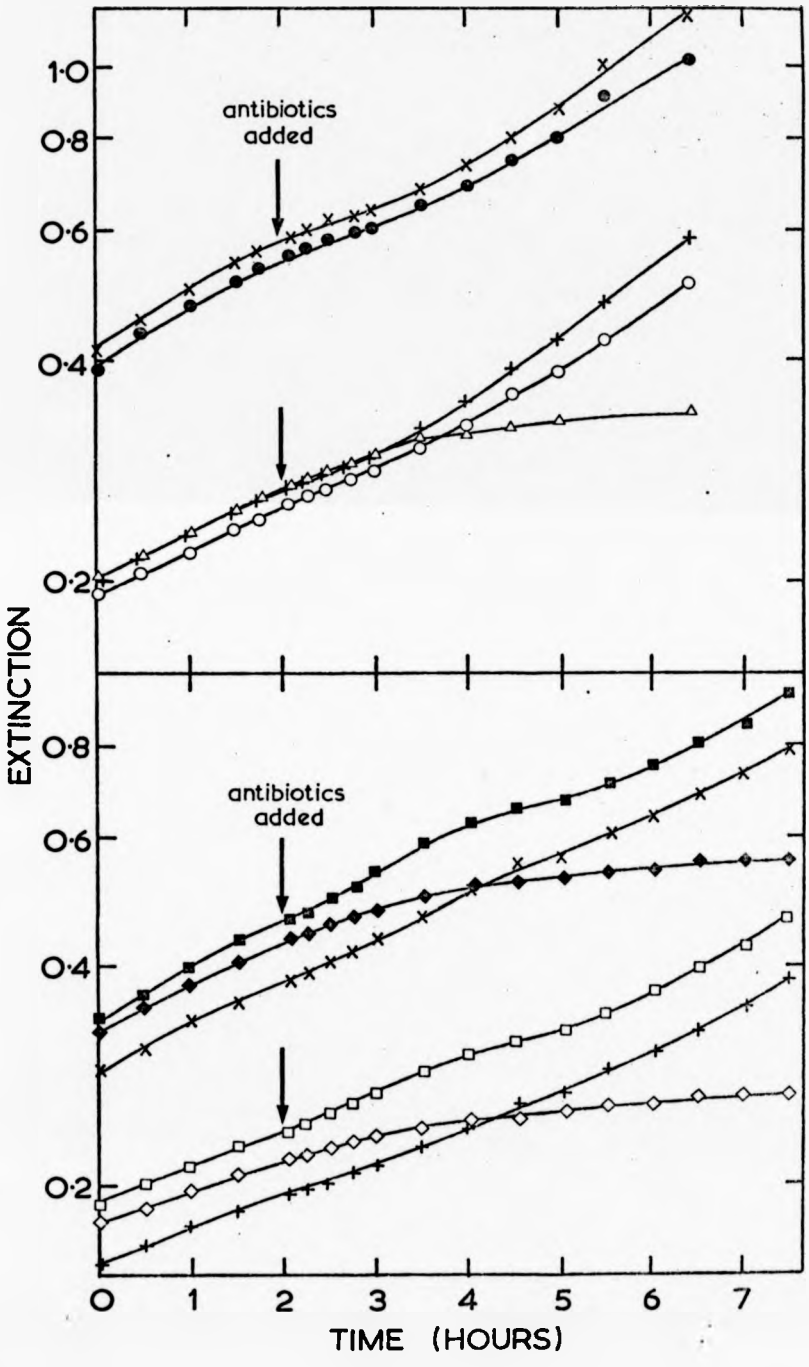


Fig. 65. Effect of antibiotics on optical density of R. palustris heterogeneous cuvette cultures. Optical densities of cultures were followed for 2 h before treating with antibiotics. Extinction of cultures at 500 nm (closed symbols and ×) and 805 nm (open symbols and +) was followed after treatment with NAL (●,○), rifampicin (△), amitrol (■,□), chloramphenicol (◆,◇), and in untreated cultures (×,+).

Fig. 65. Effect of antibiotics on optical density of R. palustris heterogeneous cuvette cultures. Optical densities of cultures were followed for 2 h before treating with antibiotics. Extinction of cultures at 500 nm (closed symbols and x) and 805 nm (open symbols and +) was followed after treatment with NAL (●,○), rifampicin (△), amitrol (■,□), chloramphenicol (◆,◇), and in untreated cultures (x,+).


1·0
0·8
0·6
0·4
0·2
antibiotics
added
0·8
0·6
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0·2
antibiotics
added
EXTINCTION
0
1
2
3
4
5
6
7
TIME (HOURS)

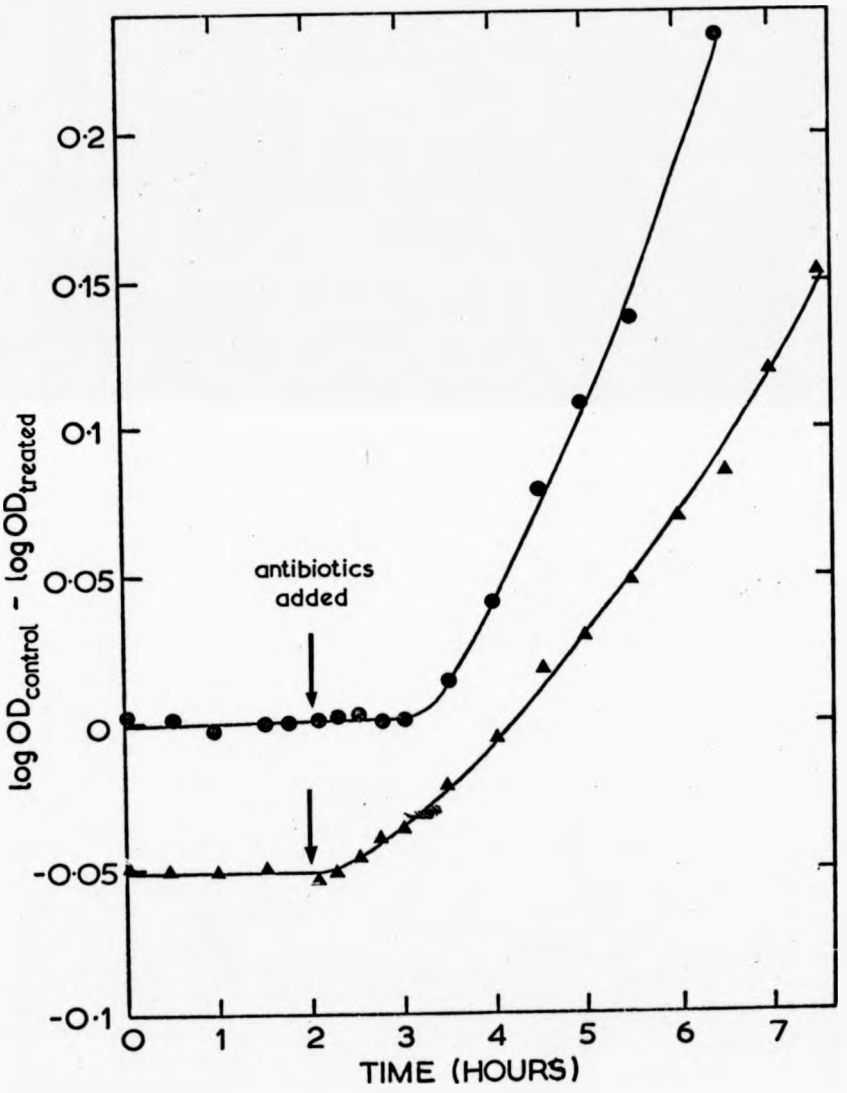

Fig. 66. Differences between untreated and rifampicin-treated (●) or chloramphenicol-treated (▲) heterogeneous culture optical densities from Fig. 65. Differences in $\log_{10}$ of extinction at 805 nm were plotted in each case.

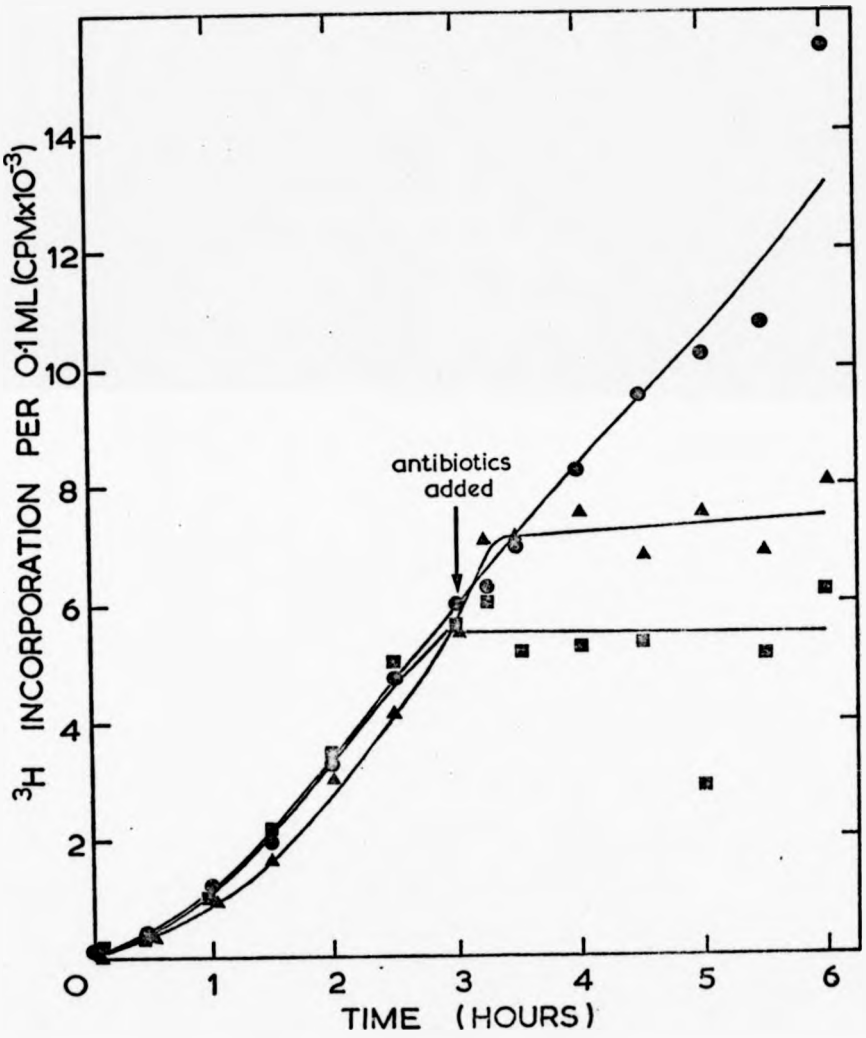

Fig. 67. Effect of rifampicin and chloramphenicol on incorporation of ^{3}H-leucine by heterogeneous populations of R. palustris. Uptake of ^{3}H-leucine was followed in an untreated culture(●) and in cultures treated after 3 h with rifampicin (▲) or chloramphenicol (■).

chloramphenicol-treated culture and indicated a delay of 0.5 h. Although this delay was shorter than that revealed by optical density changes the possibility still remained that stable m-RNA was synthesised during growth.

Inhibition of synchronous cultures by rifampicin and chloramphenicol

Since there was a possibility of long-lived m-RNA being involved in the development of R. palustris the optical density increase patterns of synchronous populations were followed as indicators of development at 34°, with rifampicin or chloramphenicol added at different times in the cell cycle. Fig. 68 shows the results of such experiments, the antibiotics being added to the parallel cultures at the beginning of swarmer maturation (0 h), during swarmer maturation (0.9 h) and at the end of tube elongation (3.2 h). In none of these cultures did rifampicin give rise to a substantially delayed inhibition of optical density increase when compared with chloramphenicol. With the exception of swarmer maturation inhibited at 0.9 h there was no indication of growth continuing to a particular developmental stage after antibiotic treatment. Even this exception was not observed in a repeat experiment. Thus no clear indication was obtained of long-lived m-RNA being synthesised during the development of swarmer populations of R. palustris.

Effects of NAL and mitomycin C on morphology and growth

Although it has already been shown that 50 μg/ml NAL had little effect on optical density of a heterogeneous cuvette culture (Fig. 65), microscopic examination of cells which had been treated for 7 h with NAL revealed a very high proportion of abnormally elongated cells (Fig. 69). In order to determine the significance of these elongated cells in relation to the normal division cycle of R. palustris growth and morphology of NAL-treated, mitomycin C-treated and untreated synchronous cuvette cultures were compared during illuminated anaerobic incubation at 34°.

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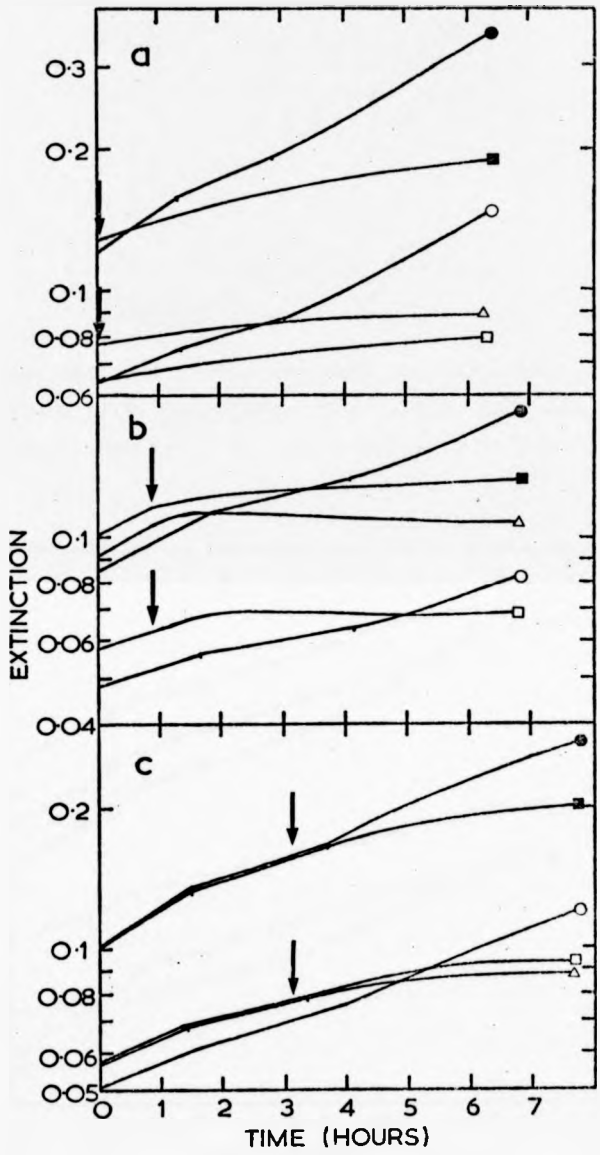


Fig. 68. Effects of rifampicin and chloramphenicol on optical densities of synchronous cultures of R. palustris. The optical density patterns at 500 nm (closed symbols) and 805 nm (open symbols) of untreated synchronous cultures (●,○) were compared with those in cultures treated at 0 h (a), 0.9 h (b) and 3.2 h (c) with rifampicin (△) or chloramphenicol (■,□).

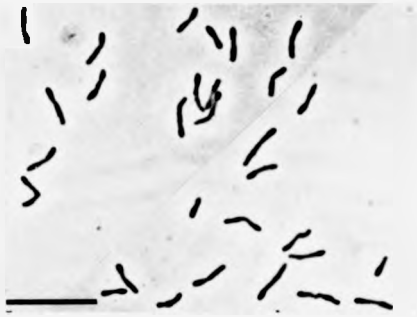


Fig. 69. Phase contrast photomicrograph of a NAL-treated heterogeneous culture of R. palustris. The culture had been grown in 50 µg/ml NAL for 7 h. Bar represents 10 µm.

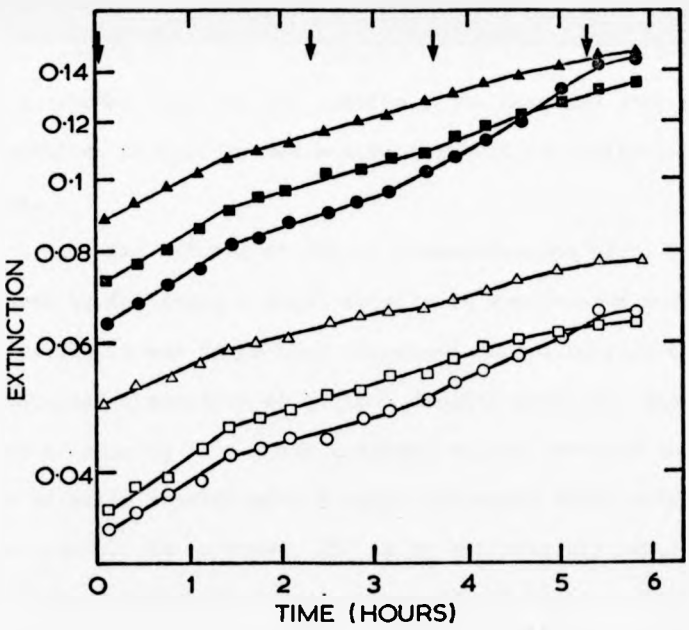


Fig. 70. Effect of NAL and mitomycin C on optical densities of synchronous cultures of R. palustris. The growth of synchronous cuvette cultures at 34° was followed by measuring optical densities at 500 nm (closed symbols) and at 805 nm (open symbols) after treatment with NAL (▲,△) or mitomycin C (■,□) and compared with an untreated control synchronous culture (●,○). Morphological development was followed and recorded photographically when indicated (↓; see Fig. 71).

Optical density changes in the cultures (Fig. 70) revealed differences between the treated and untreated cultures. Although the pattern observed was the same in all three cultures through to the end of tube elongation, beyond this stage NAL and mitomycin C gradually reduced the rate of optical density increase indicating some change in the pattern of bud maturation. Microscopic examination of the synchronous cultures (Fig. 71) showed that the presence of NAL and mitomycin C had no effect on morphological development up to the stage of bud enlargement. The normal subsequent cell division was not observed in the treated cultures. Instead, continued outgrowth of the bud occurred, the outgrown region of the cell tapering towards its growing end. The appearance of cells in the NAL-treated and mitomycin C-treated cultures was precisely the same and strongly suggested that the modified morphology was a direct result of inhibition of DNA replication.

When the effects of NAL at concentrations of 5, 50 and 250 µg/ml were compared by following optical density in synchronous cuvette cultures at 34° (Fig. 72) it was found that increased concentrations of NAL gave rise to increased inhibition of optical density changes. Microscopic examination of samples from these cultures showed there to be a high proportion of cells treated with 5 µg/ml NAL which were unaffected in their development. In contrast, 250 µg/ml NAL severely retarded development, few cells having completely formed tubes after 5 to 6 h. Only at 50 µg/ml did NAL give rise to reduced optical density increase whilst still permitting development and elongation without division. Presumably, at this concentration NAL was inhibiting DNA replication but not having any side effects on other syntheses in the cellular development.

Since developmental alterations of R. palustris induced by NAL became manifest in the latter half of the division cycle all further experiments were carried out at 36° to give rise to a more rapid development

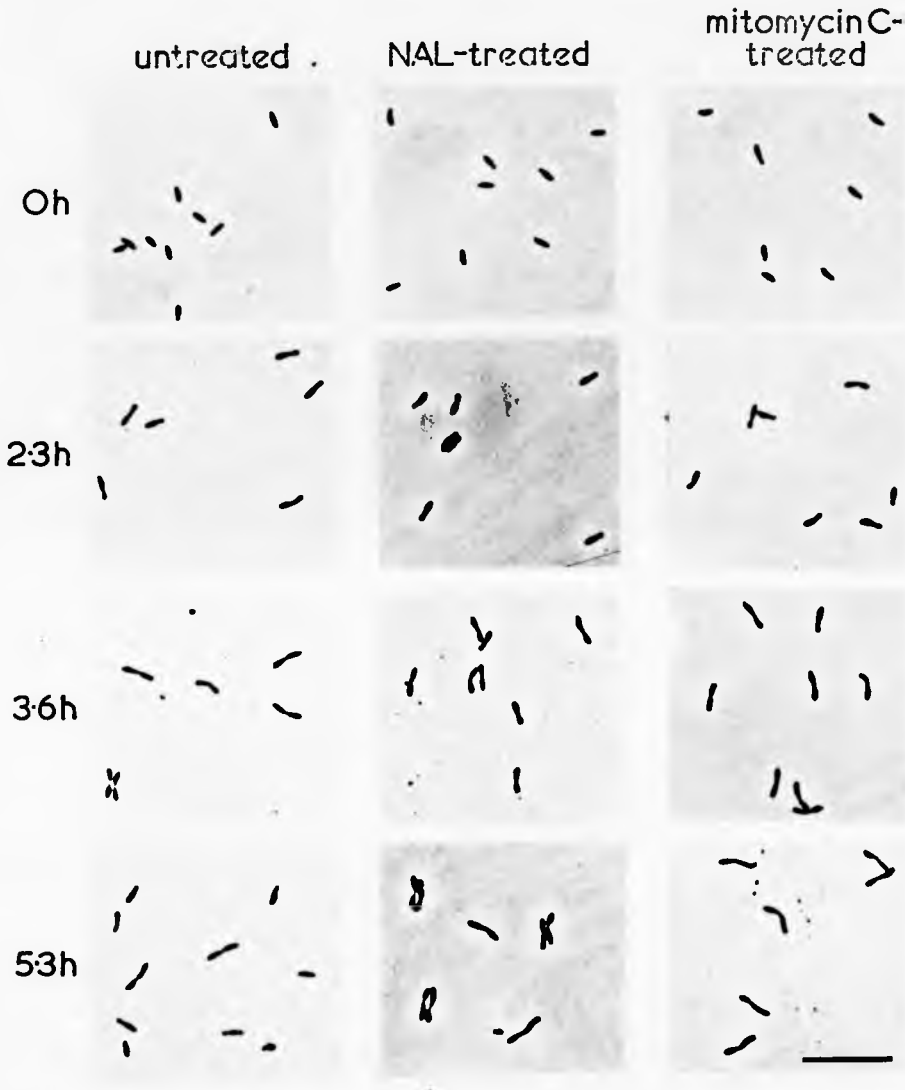


Fig. 71. Phase contrast photomicrographs of synchronous development of R. palustris in NAL-treated, mitomycin C-treated and untreated cultures. Times relate to the sampling of the cuvette cultures (Fig. 70) from which the populations were obtained. Bar represents 10 µm.

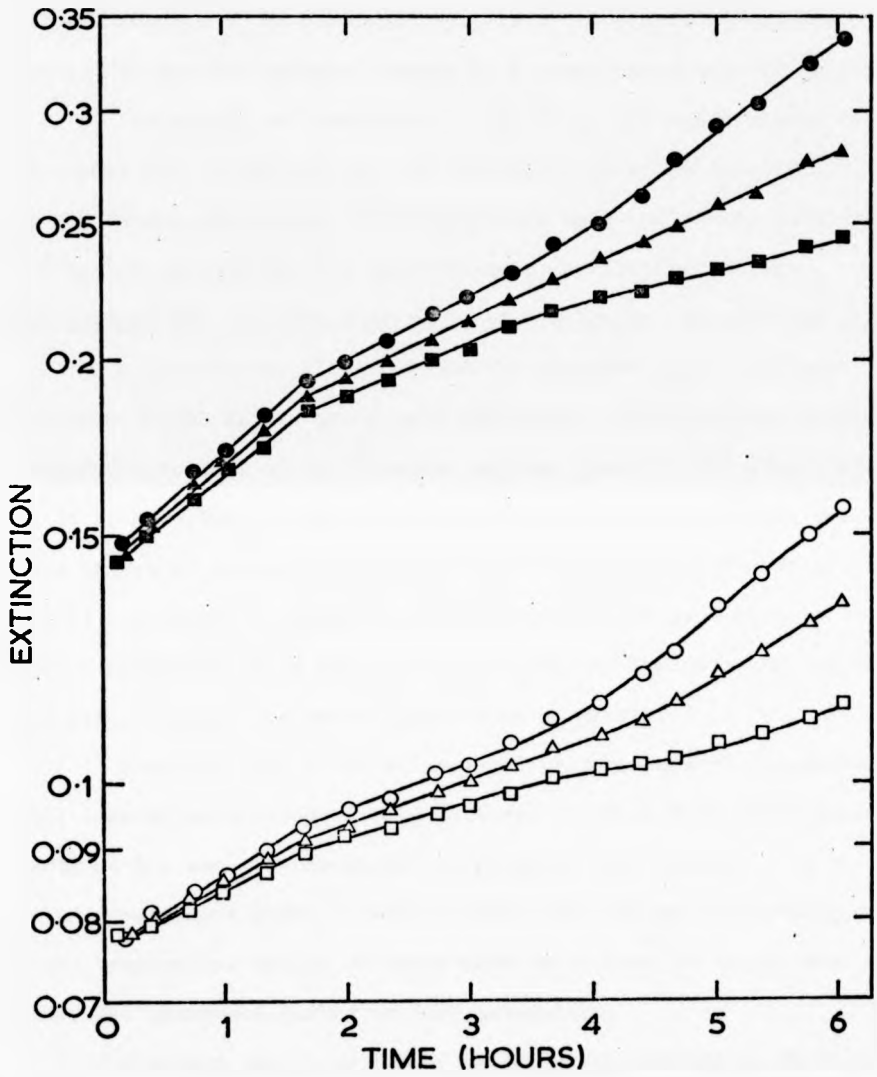


Fig. 72. Effect of different NAL concentrations on optical density of synchronous R. palustris cuvette cultures. Optical density changes were followed at 500 nm (closed symbols) and 805 nm (open symbols) in synchronous cultures at 34°, treated with 5 μg/ml (●,○), 50 μg/ml (▲,△) and 250 μg/ml (■,□) of NAL.

and allowed studies to be continued well into the second generation. Examination of optical density changes in a synchronous cuvette culture grown at 36°for nearly two generation times (Fig. 73) again showed the slight inhibition of optical density increase during bud development. During the second generation this decrease in rate continued, particularly after 6 h, but optical density increase never completely halted.

Slide culture and electron microscopy of NAL-treated synchronous cells

A synchronous slide culture was prepared using PAYE agar containing 50 μg/ml NAL as the growth substrate. Morphological development was compared with that of an untreated culture grown at 36° (Fig. 74). Growth in both cultures occurred at a similar rate and proceeded through the same series of morphological steps up to the stage of bud enlargement (4 to 5 h). Subsequent growth in the treated culture gave rise to abnormally elongated cells and no division, whilst the untreated culture divided and continued its second generation of growth.

Electron microscopy of shadowed preparations of elongated cells from NAL-treated synchronous liquid cultures revealed that cells possessed holdfasts at the non-growing mother poles (Fig. 75). However, at no time after bud development started were flagella observed on the growing bud poles and synchronous motile cultures were never seen to regain motility that they had possessed during swarmer maturation.

Although only 5 to 10% of cells in NAL-treated synchronous cultures proceeded through normal cell division, treatment for periods greater than about 10 h often gave rise to cells exhibiting a curious form of incomplete division (Fig. 76). Extensive constriction occurred along considerable lengths of the elongated cells, but division of these cells was rarely observed. These observations would seem to indicate a distinction between the narrowing of the cell diameter and the actual physical severing of the two cells, within the process of cell division.

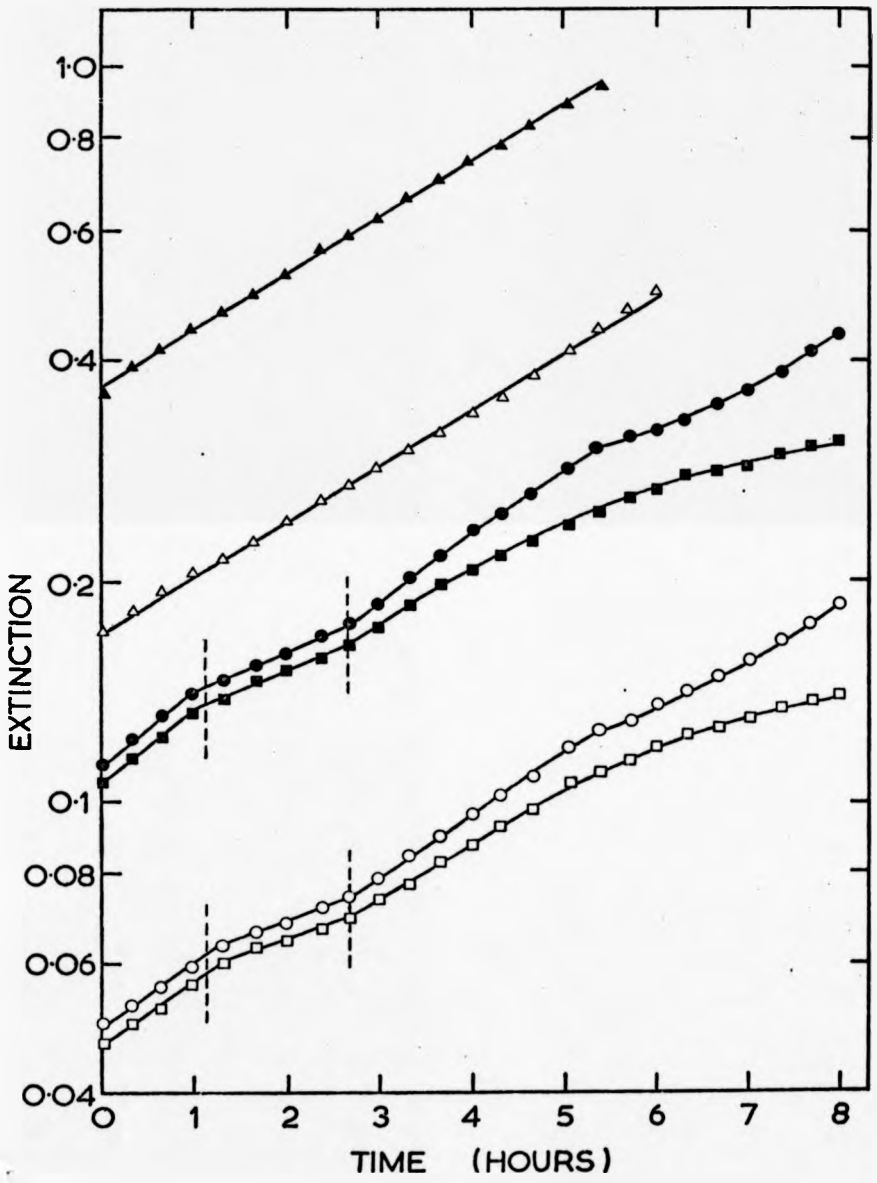


Fig. 73. Effect of NAL on optical density of a synchronous culture of R. palustris over two generation times. Cuvette cultures were incubated at 36° to enable two generation times to be examined in 8 h. Optical densities were measured at 500 nm (closed symbols) and 805 nm (open symbols) in untreated synchronous (●,○), NAL-treated synchronous (■,□) and untreated heterogeneous (▲,△) cultures.

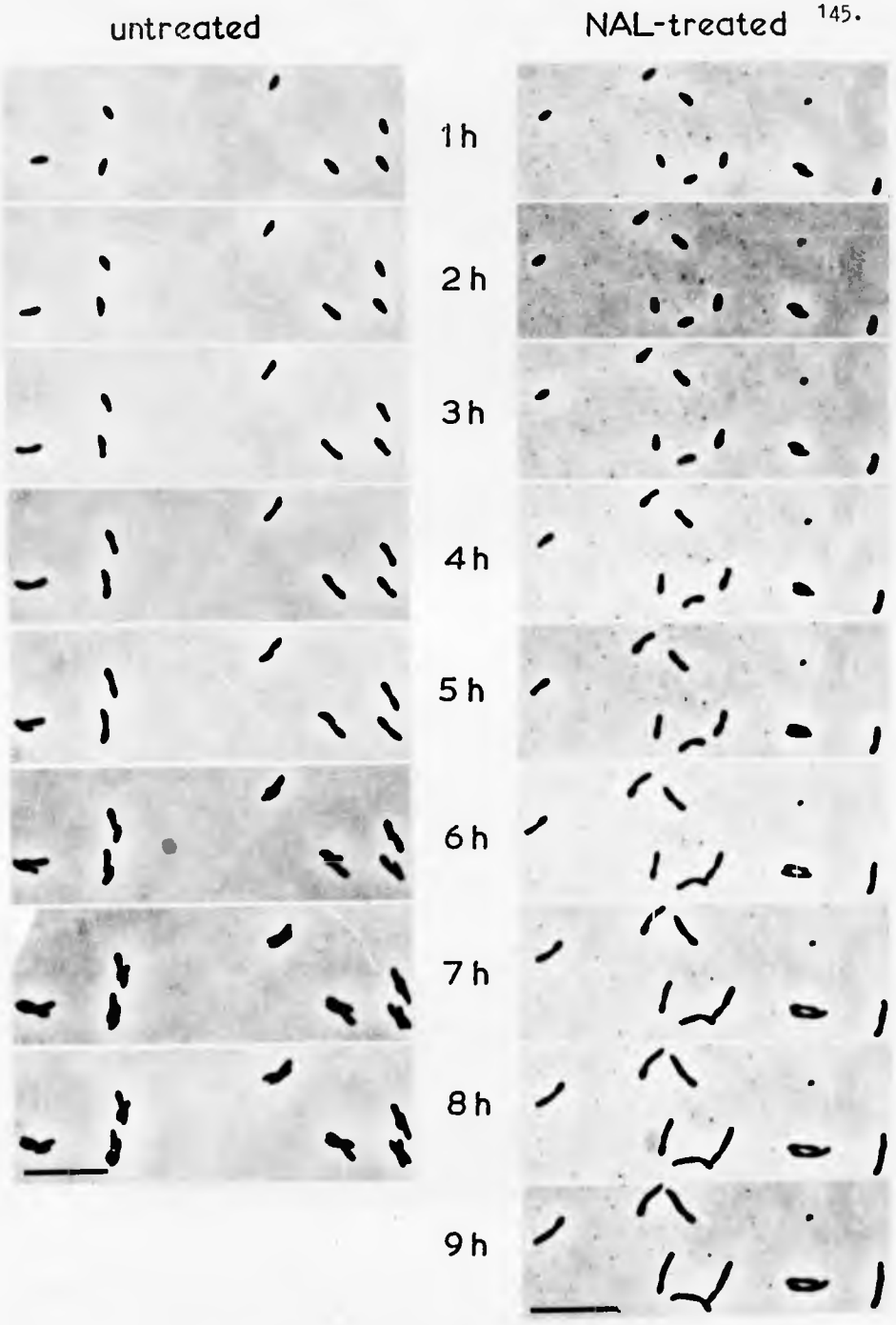


Fig. 74. Phase contrast photomicrographs of synchronous slide cultures of R. palustris, grown at 36°, showing the effect of treatment with NAL on development. Bars represent 10 μm.

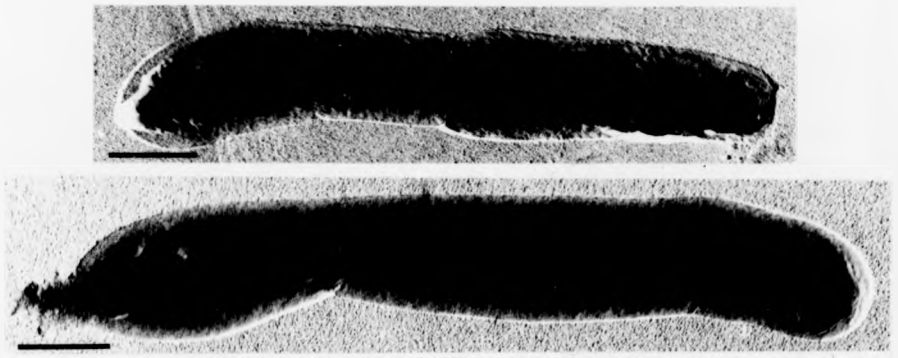


Fig. 75. Electronmicrographs of gold-palladium shadowed representatives of a NAL-treated culture of elongated R. palustris cells. Bars represent 0.5 µm.

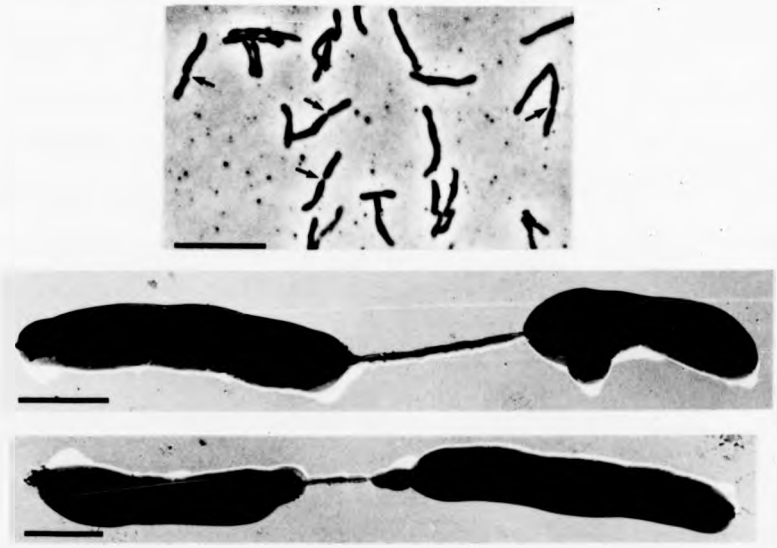
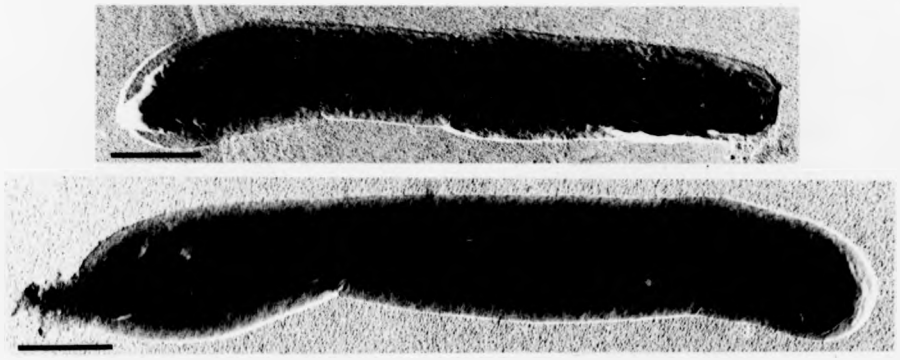


Fig. 76. Incomplete cell division occasionally observed after long-term treatment (18 h) of R. palustris with NAL. Phase contrast light photomicrograph (a), bar represents 10 µm. Electronmicrographs of gold-palladium shadowed cells (b & c), bars represent 1 µm.



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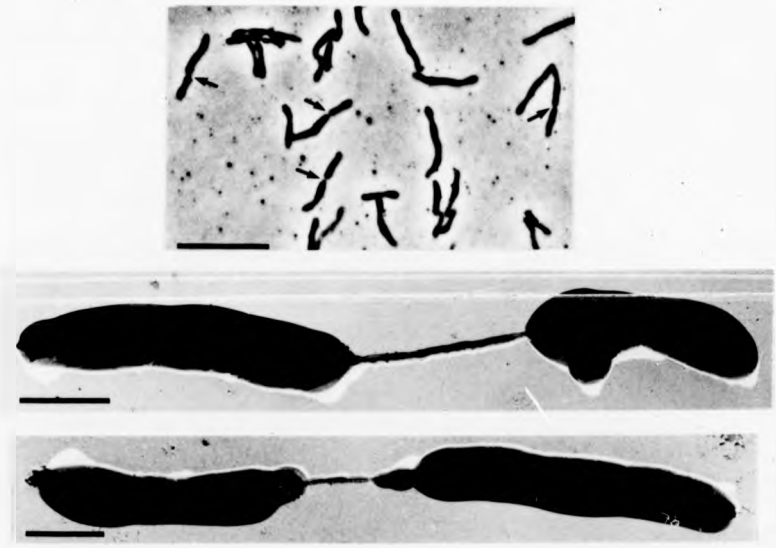

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Serial ultrathin sections through NAL-elongated cells revealed the presence of photosynthetic membrane lamellae both in the mother cell and within the elongating bud but not within the tube region of the cell (Fig. 77). The serial sections in Fig. 77 also demonstrated the relatively small area of nuclear material, compared with untreated cells just prior to division (Section IIA, Fig. 33). In general, a variety of locations of nuclear material were observed. Although they were usually relatively compact, larger fragmented nuclear bodies were sometimes observed. Apart from the more usual "loose" form of membrane lamellae which were observed, occasional cells were seen where the membranes were extremely condensed (Fig. 78a-c) and had lost the normal "triplet" double membrane appearance. It was apparent from Fig. 78a that such membrane complexes which developed in the elongating bud could extend along the full length of the cellular outgrowth beyond the normal daughter bud region.

Transverse sections of NAL-treated elongated cells revealed the normal horseshoe shape of double-layered photosynthetic membranes (Fig.79a). Occasionally cells with horseshoe-shaped condensed membranes (Fig. 79b) corresponding to those seen in longitudinal sections were also observed.

Another feature that became apparent on examination of ultrathin sections was that cells were often filled with vacuole-like areas, particularly at the elongating ends of the cells (Fig. 80b). Such "vacuoles" were also sometimes observed by light microscopy (Fig. 80a) when cells had been treated with NAL for long periods. The intricacy of such deformities within the cells suggests that the "vacuoles" were enveloped by membranes and represented a further modification of the usual ordered structure of photosynthetic membrane lamellae.

Coulter counter analysis

The effect of NAL on the increase in volume of synchronous R. palustris cultures was examined using the Coulter counter and

Fig. 77. Serial longitudinal ultrathin sections of NAL-treated elongated R. palustris cells. pm, photosynthetic membrane lamellae; n, nuclear material. Bar represents 0.2 µm.

Fig. 78a-c. Longitudinal ultrathin sections of NAL-treated elongated R. palustris cells containing condensed photosynthetic membrane structures. a & b, bars represent 0.2 µm; c, bar represents 0.1 µm.

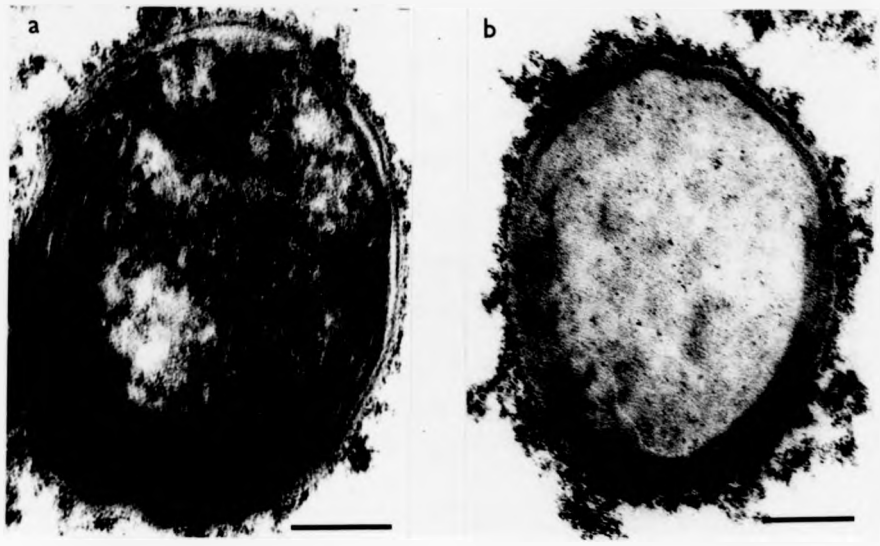


Fig. 79a,b. Transverse ultrathin sections through NAL-treated R. palustris cells. Normal double-layered membrane structure (a) and condensed membrane structure (b) are shown. Bars represent 0.1 µm.

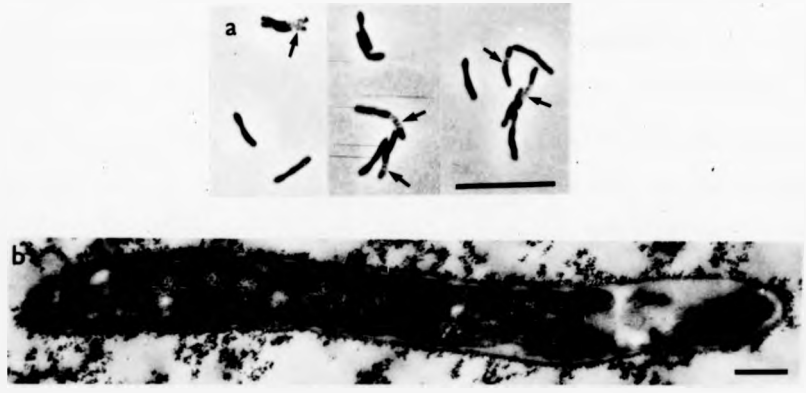


Fig. 80a,b. Vacuole-like structures in NAL-treated R. palustris cells. Cells examined by light microscopy showing "vacuoles" (↓), a; bar represents 10 µm. Longitudinal ultrathin section of cell with "vacuoles", b; bar represents 0.2 µm.

Channelyzer. Volume distributions obtained between 0 and 7 h of NAL-treated synchronous growth at 36° are shown in Fig. 81. As in untreated swarmer cultures (Section IIB, Fig. 59), selected swarmers presented a very narrow distribution of volumes, initially having a peak at about 0.26 μm^3 and gradually increasing in peak volume during incubation. However, instead of forming a double peak characteristic of cell division the cell volumes continued to increase through to the end of the experiment. After 7 h incubation the peak was becoming broader (higher standard deviation), but it indicated a mean volume of 0.89 μm^3; 3.4 times the volume of the original swarmers. This final volume represented a 40% increase in the maximum peak volume attained in 7 h by untreated synchronous cultures.

When the peak volumes shown in Fig. 81 were plotted against time (Fig. 82) an initial lag in volume increase was seen, just as in untreated synchronous cultures but followed by continuous linear volume increase. No drop in volume increase was observed over the 7 h synchronous culture in NAL. In addition, total particle counts made on dilutions of culture samples (Fig. 82) indicated no cell number increase at the expected time of division and little change of numbers elsewhere. Thus it was confirmed by Coulter counter analysis that NAL inhibited cell division in synchronous cultures of R. palustris but not volume increase, and hence growth of the cells, both before and after the normal time of cell division, was unaffected.

Synthesis of nucleic acids

The size and location of nuclear material was examined in samples of a NAL-treated synchronous culture by specifically staining DNA using the Giemsa stain method (Fig. 83). As a photographic record of the staining pattern was difficult to obtain, diagrams of the most common appearances were also prepared. Although some enlargement of nuclear bodies was observed after 1.5 to 2 h, the DNA did not seem to expand along

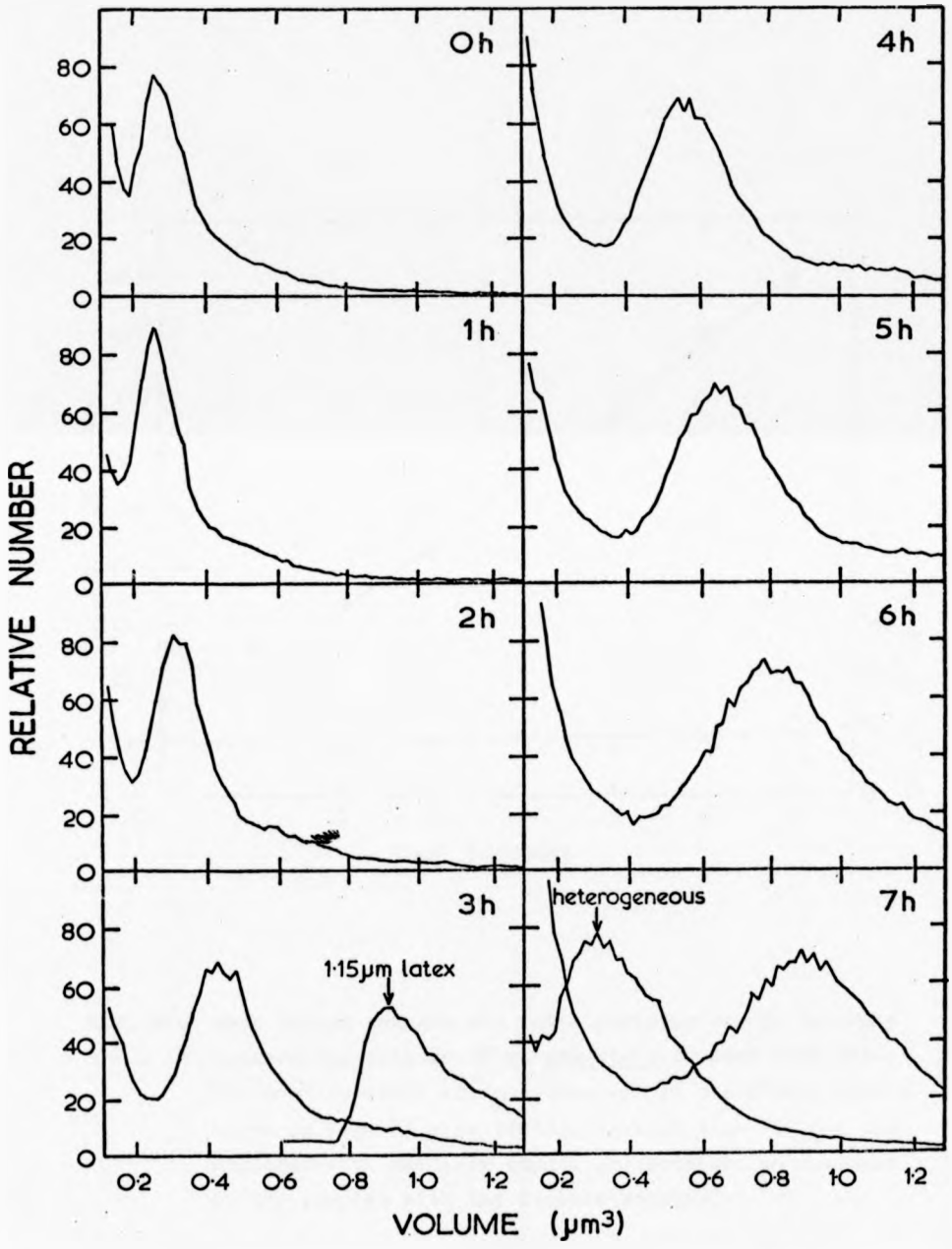
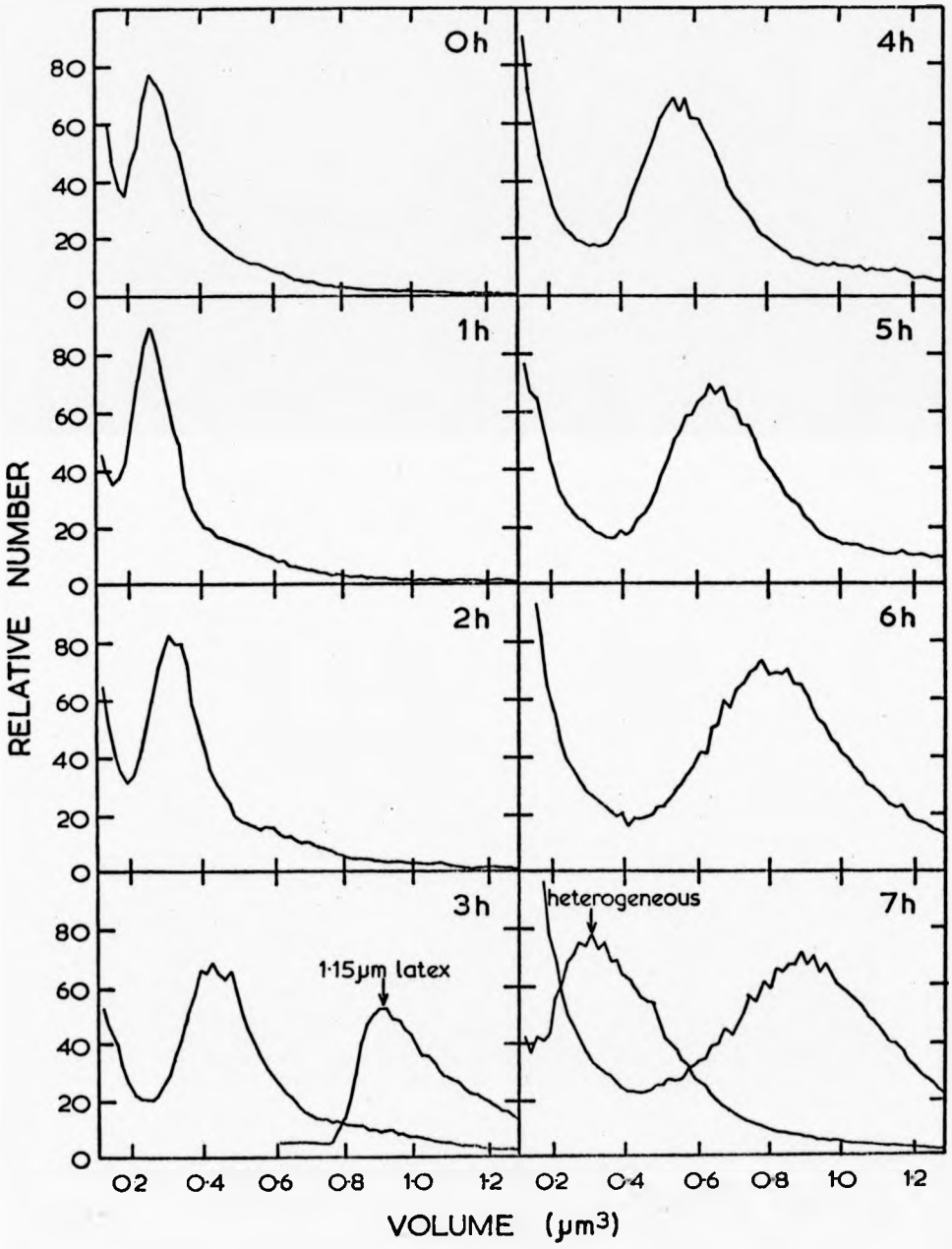


Fig. 81. Volume distributions of a synchronous culture of R.palustris treated with NAL. Samples were taken during synchronous development and volume distributions of suspended particles analysed using the Coulter counter and Channelyzer.



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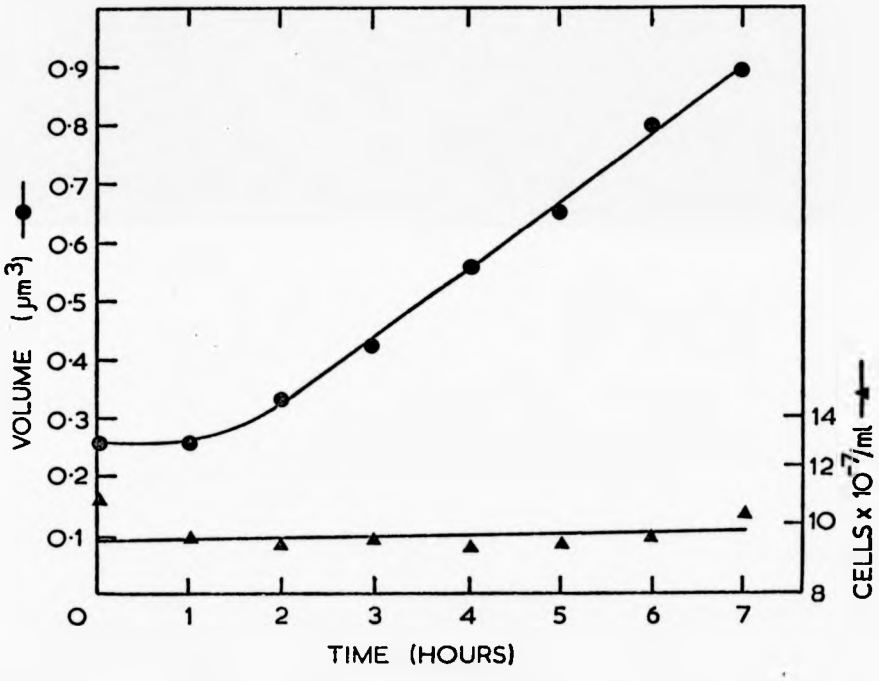


Fig. 82. Peak volume changes and total particle counts during a synchronous culture of R. palustris treated with NAL. The most frequent volumes observed in the distributions shown in Fig. 81 were plotted against time (●) and also compared with particle counts (▲) obtained on analysis of the samples with the Coulter counter.

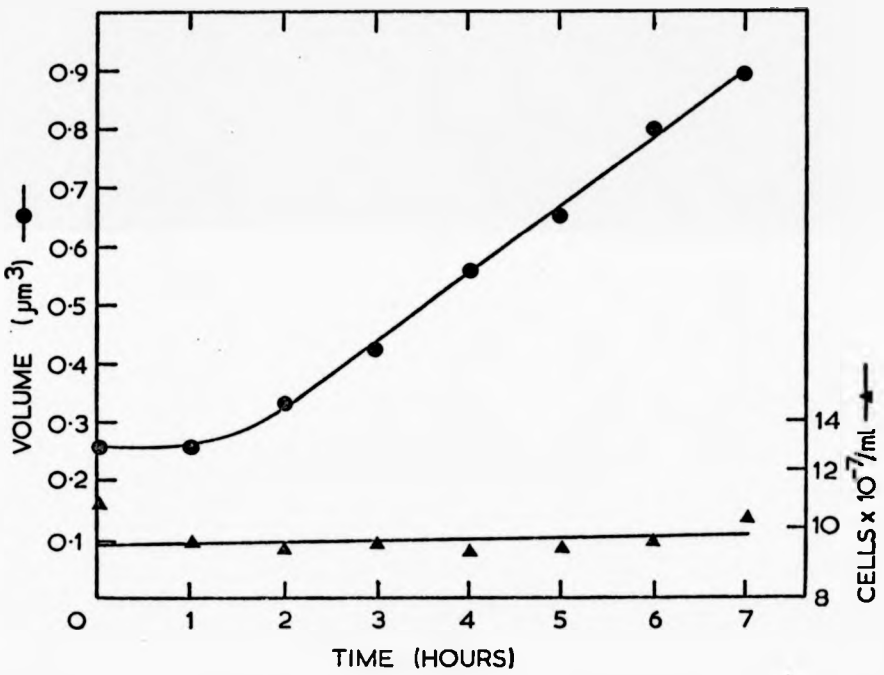


Fig. 82. Peak volume changes and total particle counts during a synchronous culture of R. palustris treated with NAL. The most frequent volumes observed in the distributions shown in Fig. 81 were plotted against time (●) and also compared with particle counts (▲) obtained on analysis of the samples with the Coulter counter.

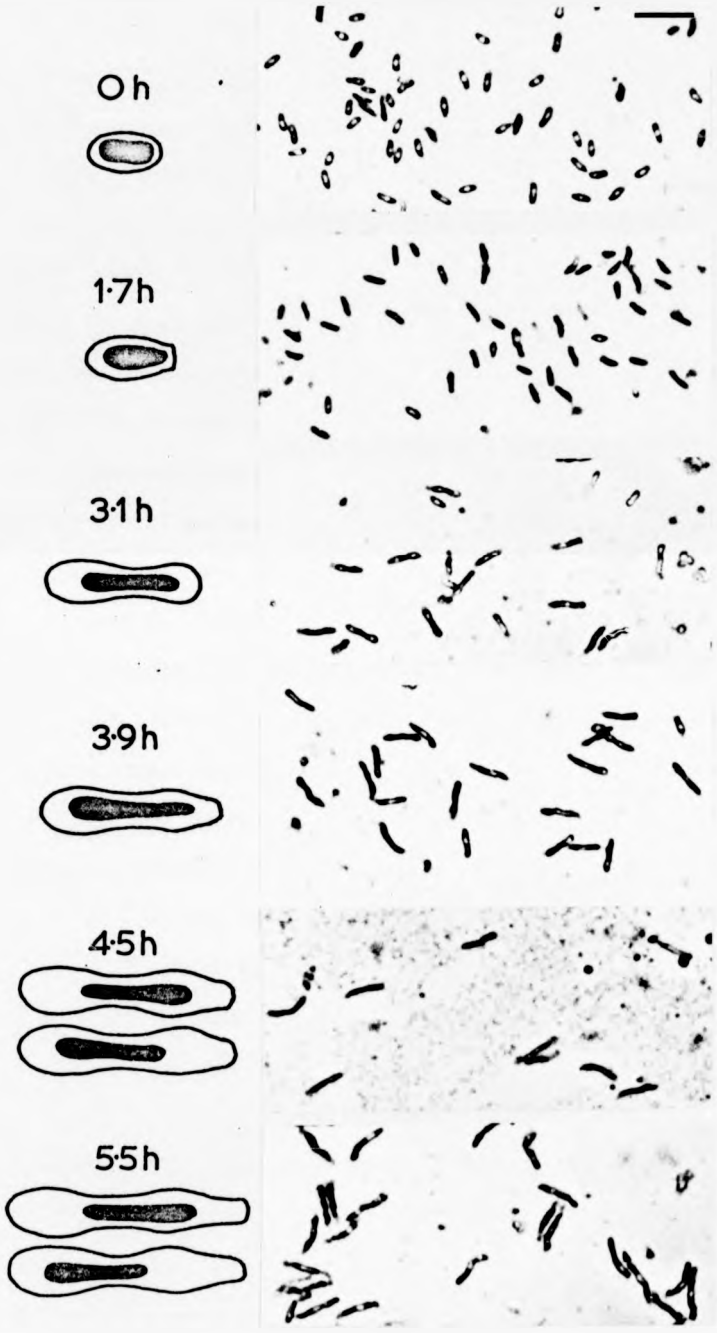


Fig. 83. Phase contrast light photomicrographs of Giemsa stained preparations of samples from a synchronous culture of R. palustris treated with NAL. Bars represent 5 µm.

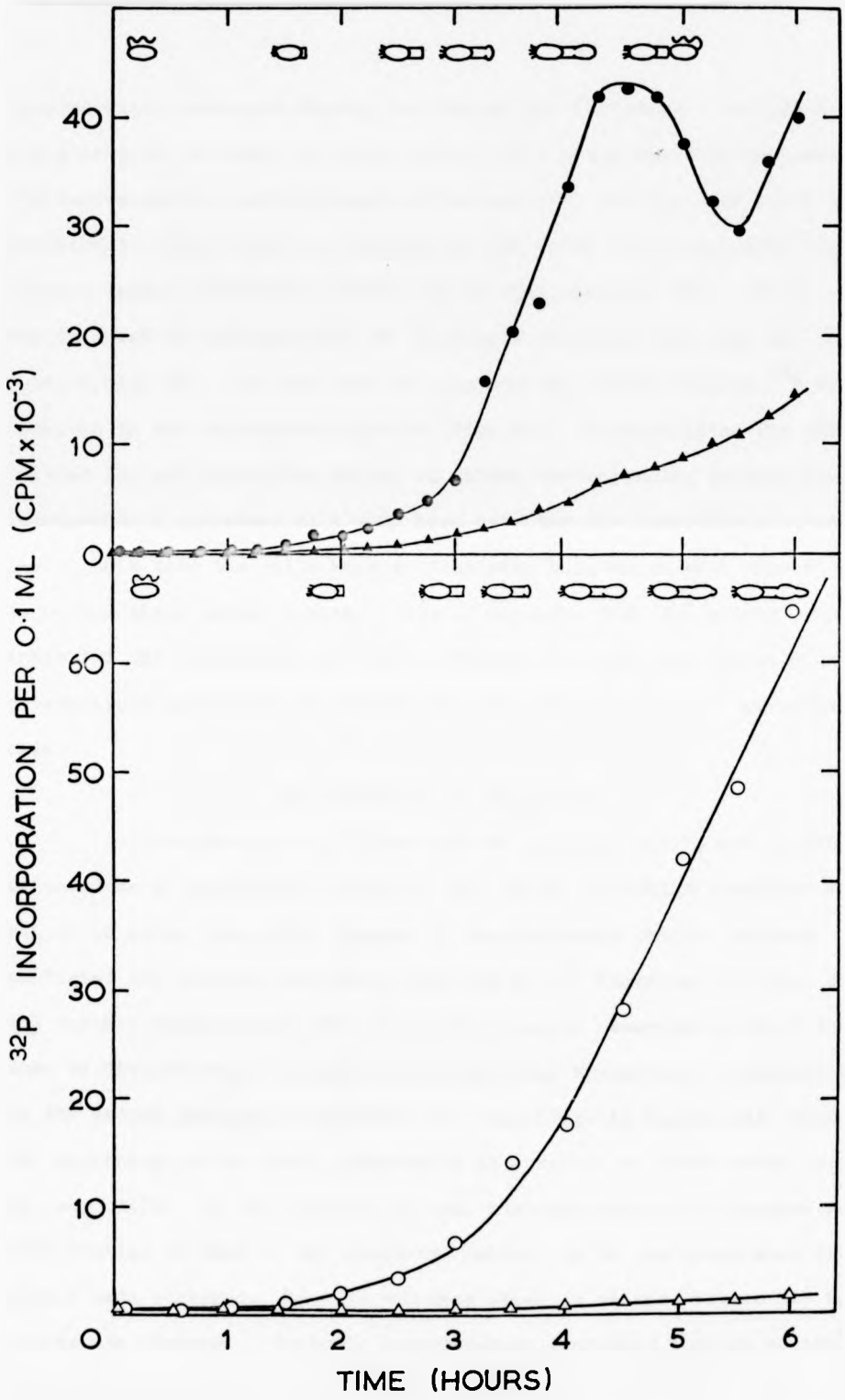
the whole length of the cell in the way that replicating chromosomes did in untreated synchronous cultures (Section IIA, Figs 33 & 35; Section IIB, Fig. 61). Thus Giemsa staining indicated that NAL was substantially inhibiting chromosome replication during the first generation. As a result of distortion of the cell outline by acid treatment it was difficult to be precise about the location of DNA within the cell. Examination of stained synchronous populations after prolonged treatment with NAL revealed that some fragmentation and maybe replication of DNA had occurred.

Incorporation of ^{32}P-phosphate into alkali-resistant and alkali-soluble fractions was followed in NAL-treated synchronous cultures of R. palustris at 36° and compared with an untreated synchronous control (Fig. 84). Levels of incorporated alkali-resistant ^{32}P in the untreated culture did not increase appreciably until after about 2 h, demonstrating that there was an initial lag in DNA synthesis during the division cycle. Although this incorporation continued into the second generation there was a temporary reduction in rate observed at 4.25 h; 0.5 to 0.75 h before most cells were dividing. In complete contrast there was little incorporation of ^{32}P into alkali-resistant fractions of the NAL-treated culture, rising only very slightly to a level of 3.2% of the total ^{32}P incorporated at 6 h. These results clearly demonstrated that NAL was inhibiting DNA replication in R. palustris.

Incorporation of ^{32}P into alkali-soluble fractions in the untreated synchronous culture (Fig. 84) showed three phases of uptake in the first generation, as recognised in the previous section (Section IIB, Fig.62). Thus negligible incorporation was detected until the end of swarmer maturation (1.25 h) when an intermediate rate of incorporation commenced and was observed for the duration of tube elongation (until about 3 h). Bud formation was then accompanied by a rapid rate of incorporation of ^{32}P into alkali-soluble fractions. Just prior to division the rate of

facing page 156.

Fig. 84. Effect of treatment with NAL on ^{32}P-phosphate incorporation by a synchronous R. palustris culture. Incorporation at 36° into alkali-soluble (●) and alkali-resistant (DNA, ▲) fractions of an untreated synchronous culture was compared with that into alkali-soluble (○) and alkali-resistant (DNA, △) fractions of a synchronous culture treated with NAL. Alkali-resistant incorporation points indicate the mean of duplicates.


40
30
20
10
0
60
50
40
30
20
10
0
1
2
3
4
5
6
32P INCORPORATION PER 0·1 ML (CPM x 10-3)
TIME (HOURS)

incorporation decreased rapidly and during the following 1 to 1.25 h there was a drop in the level of total counts. This phase was only temporary and the incorporation continued again at a high rate for the last 0.5 h of the experiment. This temporary decrease in the level of incorporated radioisotope bears a remarkable similarity to that observed when protein synthesis was followed by incorporation of ^{3}H-leucine (Section IIB, Fig. 63; this Section, Fig. 85). No such drop in incorporated alkali-soluble ^{32}P was observed in the NAL-treated culture (Fig. 84). Instead, after the usual initial lag and subsequent period of uptake corresponding to tube elongation, incorporation continued at a very high rate for the remaining 3 h incubation. During this time the cells were synthesising buds which were then elongating to beyond their normal length. Thus it appeared that NAL almost completely inhibited DNA replication but RNA synthesis, perhaps together with some phospholipid synthesis, was unaffected and continued at the pre-divisional rate.

Incorporation of ^{3}H-leucine

Incorporation of ^{3}H-leucine was followed during the growth of synchronous R. palustris cultures at 36°, with or without treatment with NAL at 50 µg/ml (Fig. 85). Uptake by the untreated control culture confirmed the pattern previously observed at 34° (Section IIB, Fig. 63) and further demonstrated that the drop in counts observed at about the time of division was substantial although only transitory. Incorporation in the second generation continued at a considerably higher rate than at the beginning of the first, presumably as a result of there being twice as many cells. In the presence of NAL, incorporation of ^{3}H-leucine was very similar to that in the untreated culture up to the stage when division should have occurred. However, at that stage no characteristic dip in counts was observed. Instead, incorporation continued through to the end of the experiment (6 h). Clearly NAL was not having any inhibitory

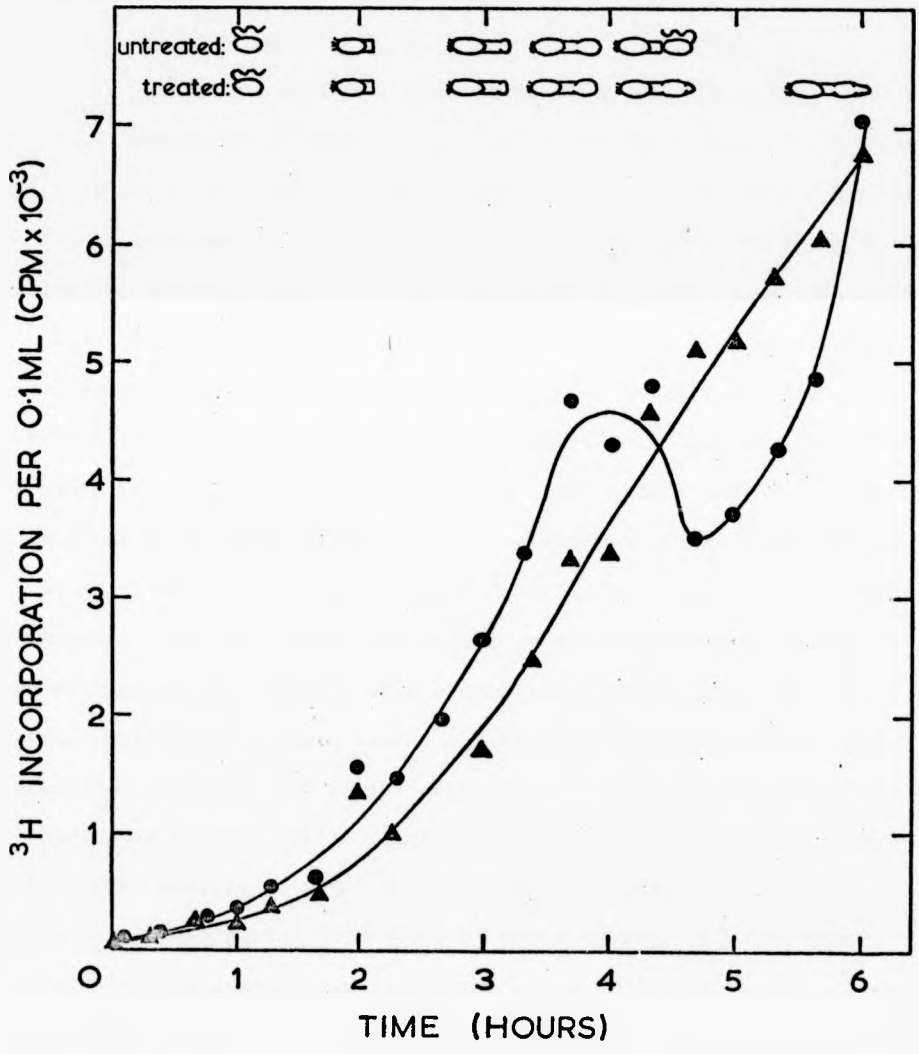


Fig. 85. Effect of NAL on ^{3}H-leucine incorporation by synchronous R. palustris cultures. ^{3}H-leucine incorporation was compared in synchronous untreated (●) and NAL-treated (▲) cultures.

effect on protein synthesis that could be detected by this means. Furthermore, the inhibition of cell division allowed continuous gross incorporation of ^{3}H-leucine to occur, showing the dip in counts in the treated culture to be directly linked to the process of cell division.

Treatment with NAL at developmental stages

Parallel synchronous cultures of R. palustris incubated at 36° were treated at different stages of development (Fig. 87) with NAL and allowed to continue to develop until cell division would normally have been complete (6 h). The proportions of elongated cells were then estimated, numbers being corrected to allow for those cells which did duplicate and thus to relate to the proportions of first generation cells affected by NAL (Fig. 86). The effect of NAL at different times of treatment was also compared with the increase in viable cell numbers (Fig. 86) obtained from a further parallel synchronous culture prepared from the same stock of selected swarmer cells. Treatment of the cells during the first 2.5 h of the cell cycle resulted in at least 90% of cells becoming elongated. By 2.95 h only half of the population produced elongated cells and microscopic examination of the cultures at about this time (Fig. 87) showed that the cells were midway through bud formation. After 3.5 h incubation more than 80% of the cells were unaffected by NAL and went on to divide as normal. Plate counts showed that cell division occurred with a mean doubling time of 4.25 h. This experiment demonstrated that there was a long period (about 1.3 h) prior to cell division when division of the synchronous population was insensitive to NAL. Since NAL has already been shown to specifically inhibit DNA replication these results would confirm that cell division in R. palustris is closely linked to DNA synthesis and that at 2.95 h the particular event or time during DNA replication (probably termination) that triggers cell division had been passed.

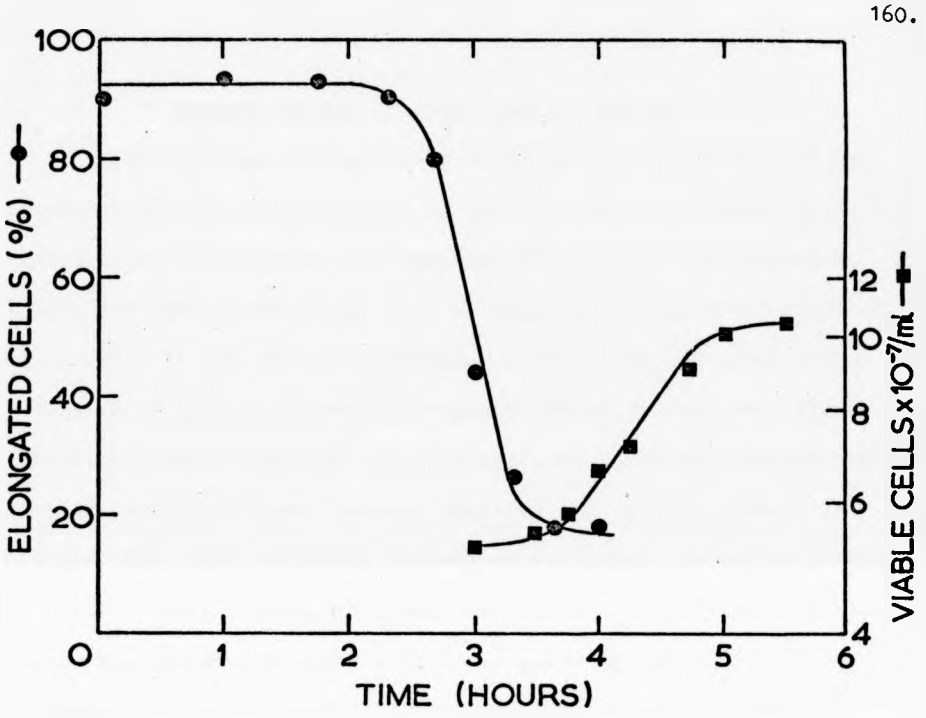


Fig. 86. Effect of NAL treatment commenced at time intervals in the division cycle of R. palustris. The proportion of elongated cells present after 6 h, resulting from treatment at times during the growth of synchronous cultures, was determined and corrected for the estimated number of cells having divided (●). Viable cell numbers in an untreated synchronous culture were determined by the spread plate method (■).

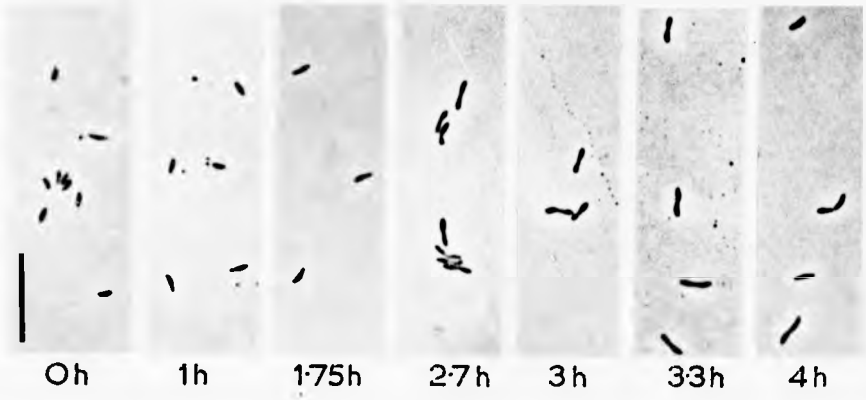


Fig. 87. Phase contrast photomicrographs of cell samples at the times of NAL treatment in Fig. 86. Bar represents 10 μm.

Removal of NAL at developmental stages

A synchronous culture was treated at zero time with NAL and then samples taken at various stages of the subsequent development at 36°. Immediately the samples were serially diluted and transferred to pre-warmed, nitrogen-gassed PAYE, thus allowing continued development of the sample cells in the effective absence of NAL. Fig. 89 shows cells representative of the developmental stages at which the NAL was diluted out. Plate counts were carried out on samples of these NAL-free sub-cultures and plots of doubling of cell numbers obtained during the division of the cultures (Fig. 88). Although removal of NAL after 1 h had no effect on the doubling time, removal at times later in the cell cycle gave rise to progressively longer doubling times. By plotting the difference between mean doubling times of treated samples and the effectively untreated control against the time of diluting out the NAL (Fig. 90) it was possible to demonstrate that there was no delay in cell division when synchronous cells were treated with NAL up to 1.3 h. This was about 2.75 h before cell division occurred in the control culture and corresponded approximately to the beginning of tube elongation. It was also shown that the subsequent delay in cell division was directly proportional and almost equal to the time after 1.3 h that NAL was diluted from the culture. Clearly NAL had no inhibitory effect on cell division during the first 1.3 h of the cell cycle.

Outgrowth during reversal of NAL treatment

A synchronous culture of R. palustris was treated with NAL and incubated at 36°until abnormal cell elongation was detected (5 h). The cells were then washed twice with PAYE and used as an inoculum for a 36° slide culture (Fig. 91). Incubation of the cultures was commenced 6 h after the NAL treatment of the synchronous culture had been initiated.

Three alternative patterns of development were seen in the

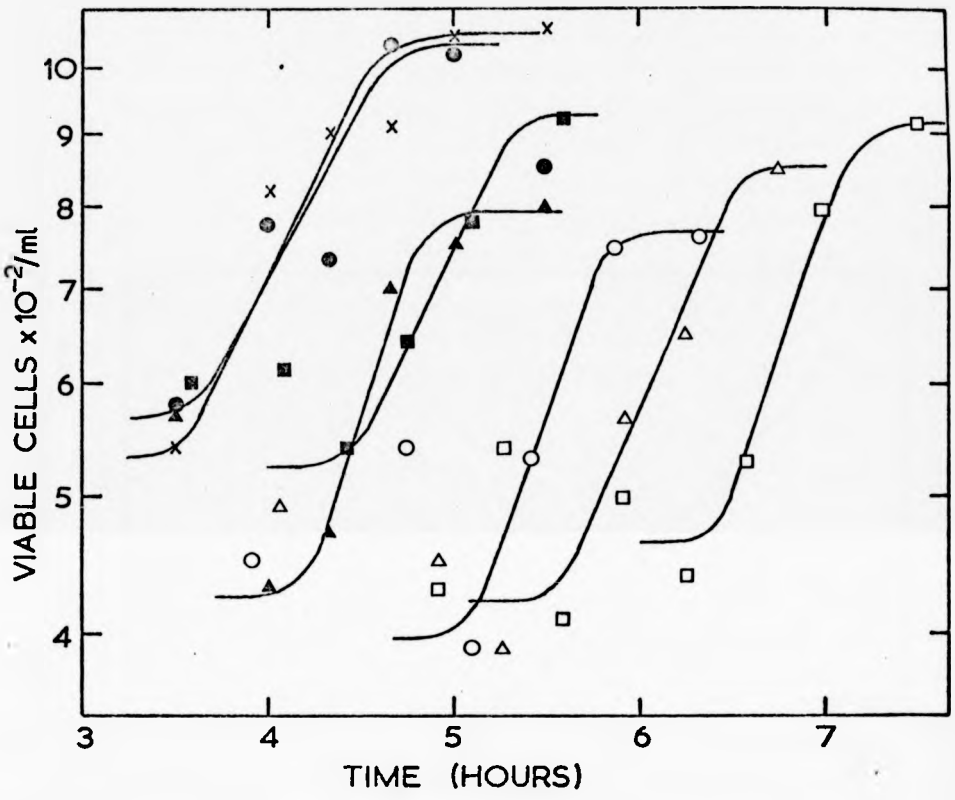


Fig. 88. Effect of stopping NAL treatment at time intervals in the division cycle of R. palustris. Samples of a NAL-treated synchronous culture were taken at intervals, diluted 10^5-fold and incubation continued. Plots of viable cell numbers in the resulting cultures were obtained for samples diluted at 0 h (x), 1 h (●), 1.75 h (▲), 2.25 h (■), 2.75 h (O), 3.25 h (△)and 3.75 h (□).

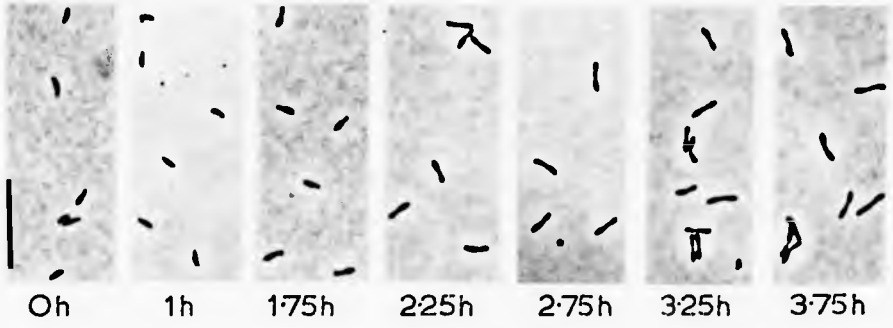


Fig. 89. Phase contrast photomicrographs of cell samples taken at the same time as samples diluted to stop NAL treatment in Fig. 88. Bar represents 10 μm.

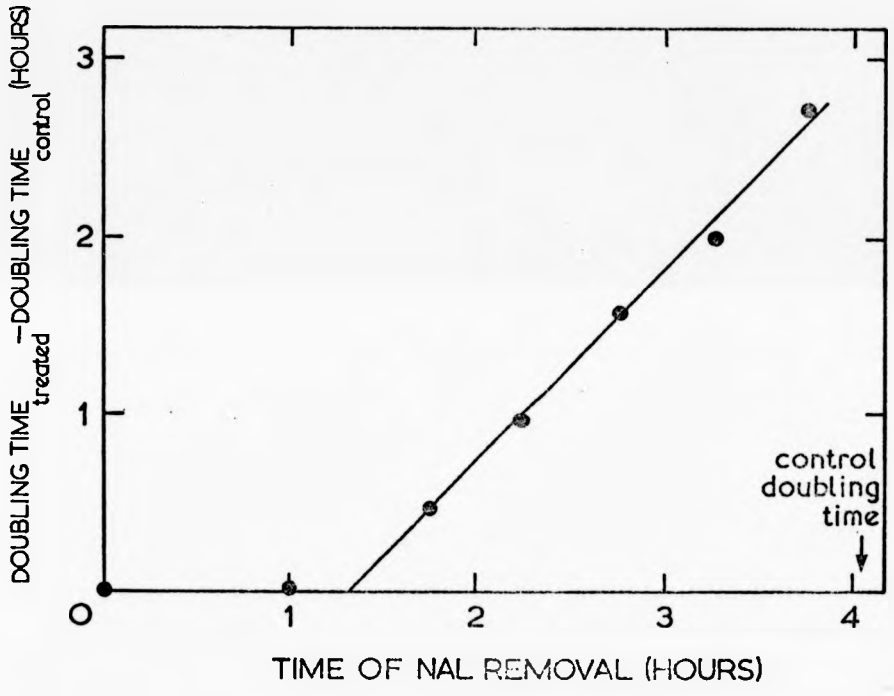


Fig. 90. Delay in doubling time observed in NAL-treated synchronous R. palustris culture where NAL was effectively removed by dilution at time intervals in the division cycle. The doubling time of a culture diluted at 0 h (as indicated) was considered as the untreated control and was subtracted from doubling times obtained for other times of diluting out NAL (see Fig.88).

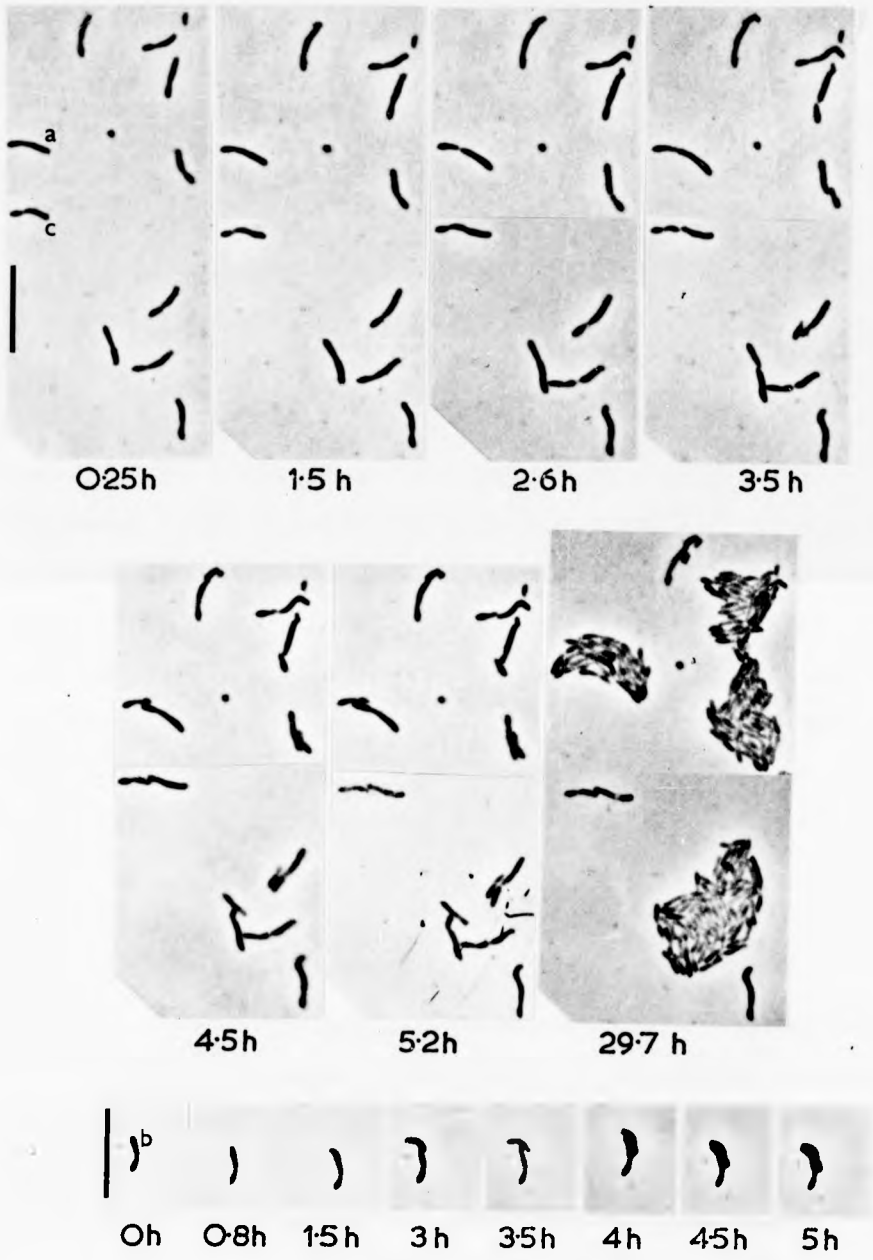


Fig. 91. Phase contrast light photomicrographs of growth of R. palustris after removal of NAL from elongated synchronous cultures. Synchronous cultures were grown for 5 h in the presence of NAL, were then washed and incubated as slide cultures free from NAL. The start of the slide cultures (6 h from start of synchronous culture) was considered as 0 h. a, b & c indicate the three responses of cells to reversal. Bars represent 10 µm.

outgrowth of the cells. Most commonly there was an initial lag of 1 to 1.5 h followed by continued cell elongation over the next 1.5 h after which time complete cell division was seen between the end of the normal mother cell tube and the grossly elongated daughter cell (Fig. 91, cell a). Both cells were capable of further development and gave rise to microcolonies on the slide cultures. Alternatively, after a period of outgrowth, budding was occasionally observed at the end of the cell distal to the holdfast pole (Fig. 91, cell b). The third pattern of development was a continued elongation of the cell followed, after a variable period of time, by complete or incomplete division part way along the swollen elongated bud (Fig. 91, cell c). However, this form of development resulted in two non-viable post-divisional cells. This effect gave rise to very similar progeny cells to those in the incomplete division sometimes seen during long-term treatment of cells with NAL (Fig. 77).

DISCUSSION

Growing R. palustris cells treated with penicillin clearly revealed the polar, unidirectional nature of wall growth in this organism. Unidirectional growth of the wall of E. coli, cultured on minimal medium, has similarly been demonstrated by penicillin treatment (Donachie & Begg, 1970). It is important to note that this unidirectional growth in E. coli was **not** the same as polar growth in budding bacteria such as R. palustris. In E. coli the cell wall growth point remained at a fixed distance (unit cell length) from the "old" cell pole, the new wall material extending beyond this growth point(Fig. 92a). The growth point was then already in the correct location for cell division when two unit cell lengths were reached. During elongation of R. palustris the cell wall growth

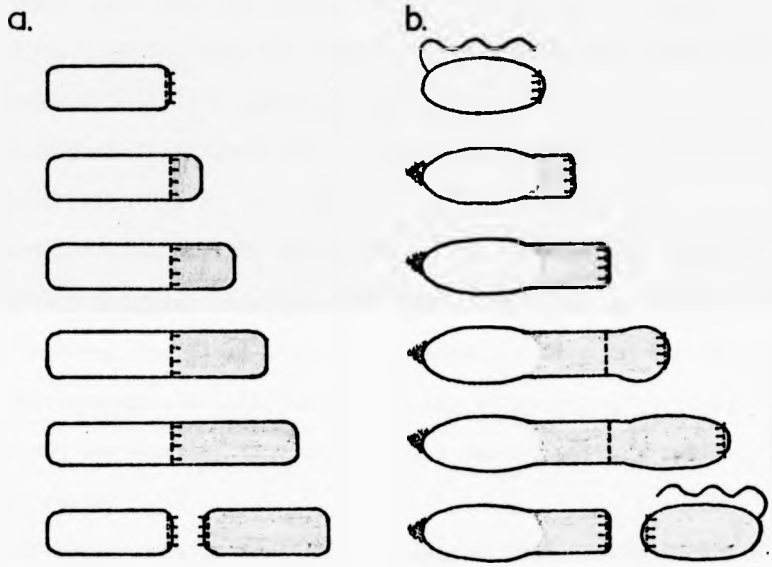


Fig. 92. Unidirectional cell wall growth models in the cell cycles of E. coli (a) (as proposed by Donachie & Begg, 1970) and R. palustris (b). Vertical dashes indicate potential growth regions; horizontal dashes indicate direction of laying down wall material from growth region.

point was always at the tip of the cell, new wall material being laid down behind the growing tip (Fig. 92b). However, just prior to division there is a very important change in the location of active wall growth, from the tip to a predetermined site at the division plane. Together with all other organisms exhibiting true polar growth, R. palustris provides an undoubted exception to the generalisation put forward by Donachie & Begg (1970) that penicillin-sensitive sites and potential division sites occupy identical positions in the cell.

At approximately the same time as wall growth stops at the extreme tip of the daughter cell the flagellum develops. At a later stage the holdfast replaces the flagellum at this same location on the daughter cell wall. Consequently, within the cell cycle of R. palustris there is another localised sequence of irreversible and specifically timed developmental events.

Jacob, Brenner & Cuzin (1963) suggested that membrane growth between points of attachment of chromosomes could provide a primitive "mitotic" system for bacteria. Donachie et al (1973) have pointed out that their model correlating initiation of DNA replication with multiplication of envelope growth sites provides an extention of this hypothesis. Since there is little or no cell envelope growth at the potential division plane prior to division it is impossible to apply this mechanism to an organism such as R. palustris.

In their study of Caulobacter and Asticcacaulis, Schmidt & Stanier (1966) demonstrated that the organisms had two growth points; one at the division plane for cell replication and another at the junction of the cell and stalk for stalk elongation. Thus the stalk of Caulobacter differs from the tube of R. palustris not only in being a non-reproductive prostheca but also by extending by basal growth rather than polar growth.

It is probable that the composition of the cell wall synthesised

during the division cycle of R.palustris is slightly altered at specific times giving rise to the changes of cell shape that are observed. Such alteration of wall composition giving rise to changes in cell shape has been well documented for the sphere-rod morphogenesis exhibited by Arthrobacter crystallopoietes (Krulwich et al, 1967a,b). Although control of wall composition in this organism is known to respond to changes of growth rate (Luscombe & Gray, 1971) it is quite possible that ordered control of changes in wall composition could occur during the cell cycle of R. palustris. Perhaps an analysis of wall composition of swarmers compared with developing cells or heterogeneous populations might reveal these slight differences.

Rifampicin treatment of heterogeneous cultures of R. palustris inhibited optical density increase and protein synthesis only after a delay. Since the delay was greater in the former it could in part be accounted for by relatively slow decay rates of enzymes synthesised just before inhibition. Although delays were also observed in the inhibitory action of chloramphenicol on optical density increases, such delays were not observed in the inhibition of protein synthesis. The delayed effect of rifampicin in comparison with chloramphenicol could have been due to low permeability of cells to rifampicin or maybe to the synthesis of stable m-RNA. Unfortunately, such delays could not be demonstrated during the course of synchronous growth of R. palustris. The optical density method of following development of cultures may not have been sensitive enough, particularly when extinction values obtained for rifampicin-treated cultures were very low. It is also possible that stable m-RNA might be synthesised on few occasions in the developmental cycle and that these were missed in the synchronous culture experiments. An investigation of the effects of rifampicin and chloramphenicol on occurrence of specific events such as loss of motility, synthesis of holdfast and de novo membrane synthesis might give more meaningful results.

The morphological development of R. palustris swarmer cells in the presence of NAL was normal through to the end of bud growth when the cells continued to grow and elongate instead of dividing. During the course of the synchronous development synthesis of DNA was almost completely inhibited. ^{32}P incorporation into alkali-soluble fractions was unaltered up to the completion of bud formation, when compared with untreated cells, and was in no way inhibited during the abnormal elongation. It is generally accepted that NAL has little effect on RNA synthesis (Goss et al, 1964; Winshell & Rosenkranz, 1970). Nevertheless, when net protein synthesis of E. coli was inhibited Javor (1974) found that NAL substantially inhibited RNA synthesis. NAL has also been found to inhibit RNA synthesis in C. crescentus (Degnen & Newton, 1972b) but in this case there was only partial inhibition of DNA synthesis.

Because NAL has little effect on incorporation of certain amino acids (Goss et al, 1965) it is generally assumed that it does not significantly inhibit protein synthesis. However, it is important to note that Shuman & Schwartz (1975) recently demonstrated that synthesis of certain inducible enzymes of E. coli K-12 is directly affected by NAL. The pattern of ^{3}H-leucine incorporation exhibited by developing R. palustris swarmers indicated that protein synthesis was unaffected by NAL, except that incorporation patterns relating to cell division were not observed. The uptake of ^{3}H-leucine was sensitive to inhibitors of m-RNA synthesis; further evidence that RNA synthesis in R. palustris was unaffected by NAL.

The characteristic decrease in levels of incorporated alkali-soluble ^{32}P and ^{3}H-leucine were not observed in the NAL-treated cultures suggesting them to result directly from cell division or DNA replication. One possible explaination of such results is that there is either a leakage or excretion of RNA and protein during cell division. Alternatively, both RNA and protein turnover could increase dramatically rendering the

otherwise insoluble incorporated material soluble in cold TCA. The significance of these observations is not known, but it is possible that such losses may be concerned with very rapid synthesis and excretion of flagellin.

Volume distributions of NAL-treated cells demonstrated that there was an uninterrupted linear volume increase even beyond the normal time of cell division. The volume increase can be considered as being dependent on the continued elongation of cells. Very similar observations were made by Clark (1968) with E. coli synchronous cultures treated with NAL. E. coli is not the only other organism where cell elongation in response to treatment with NAL has been observed. Blackman & Weiner (1975) examined individual H. neptunium cells from heterogeneous populations supported on an agar film and observed that both bud formation and release (cell division) were suppressed by NAL. Filaments that were produced by treated swarmer cells continued to elongate beyond their normal maximum length, but few swarmers produced daughter buds.

When DNA synthesis in C. crescentus was inhibited by mitomycin C or hydroxyurea Degnen & Newton (1972b) found filamentous cell formation to be induced. Cell division in C. crescentus is normally accompanied by stalk elongation and crossband formation (Staley & Jordan, 1973). Haars & Schmidt (1974) demonstrated in mutants which produce long stalks that stalk elongation is inhibited by mitomycin C and hydroxyurea. This demonstrates another difference between the stalk of Caulobacter and the tube of R. palustris.

Since photosynthetic membranes were observed in buds of R. palustris developing in the presence of NAL it would seem that their de novo synthesis in a specific location is not triggered or controlled by the daughter genome whilst it is being synthesised. Instead, it must be ordered by transcription of the mother cell genome at a specific time.

In this way photosynthetic membrane synthesis is independent of chromosome replication. The intracellular location of the new photosynthetic membrane lamellae must also be controlled by some means. This control may be related to a growth region which remains at the end of the tube, where membrane synthesis can continue but subsequent wall synthesis leading to division would be inhibited by NAL. Although photosynthetic membrane synthesis and cell wall elongation were apparently independent of chromosome replication, synthesis of flagella and holdfasts on the developing bud cells was inhibited by NAL, in addition to cell division. This suggests a common control of these three phenomena, linked to the termination of chromosome replication.

When NAL was added to synchronous cultures of R. palustris at different developmental stages it was found that there was a period of 1.3 h prior to cell division when division of the populations was insensitive to NAL. This indicated that chromosome replication was completed at the beginning of the period and that at the same time cells became committed to divide. When DNA synthesis was followed in the cell cycle by incorporation of ^{32}P there was no complete interruption in synthesis apparent at this time. This may have been due to the insensitivity of the method of following DNA synthesis or to the continued synthesis of a new round of chromosome replication in the mother cell.

The pattern of chromosome replication in several other organisms has been shown to be intimately related to cell division. Cooper & Helmstetter (1968) and Helmstetter & Cooper (1968) showed that in rapidly growing E. coli DNA synthesis occurred at a constant fixed rate, chromosome replication therefore taking a fixed time at a given temperature (the C period). There was also a constant period between the end of one round of chromosome replication and cell division (the D period). Studies on the effect of NAL on the division cycle of E. coli have clearly

demonstrated that inhibition of DNA synthesis during the D period has no inhibitory effect on subsequent cell division, indicating a controlling link between completion of chromosome replication and cell division (Helmstetter & Pierucci, 1968; Clark, 1968; Dix & Helmstetter, 1973; Burdett & Murray, 1974).

Blackman & Weiner (1975) found that half the H. neptunium cells which they examined that initially had buds proceeded to divide even though treated with NAL. They suggested that division might be triggered part way through bud formation and that this may signify completion of chromosome replication. Indeed, Moore & Hirsch (1973b) reported that DNA replication in Hyphomicrobium strain B-522 did occur during development preceeding the beginning of bud formation.

The pattern of chromosome replication in C. crescentus swarmer cells has been established as consisting of an initial lag, a synthetic period and a pre-divisional lag (Degnen & Newton, 1972a). Again, cell division has been found insensitive to DNA synthesis inhibitors during the period between completion of chromosome replication and cell division (Degnen & Newton, 1972b).

Further evidence confirming the presence of the initial lag in DNA synthesis in R. palustris was provided by the experiment where timing of cell division was followed after NAL treatment had been stopped at different times in the cell cycle. Since NAL specifically inhibits DNA replication, and cell division processes are probably temporally linked to the completion of chromosome replication, the time pinpointed in the experiment almost certainly corresponded to the initiation of chromosome replication. The delay of cell division by treatment with NAL was due to initiation of DNA replication being delayed beyond its normal starting time. The experiment depended on the reversible nature of NAL action as well as immediate continuation of any DNA synthesis after

removal of the inhibitor. Indeed, Ward et al (1970) have demonstrated that chromosome replication in E. coli was initiated 3 min after removal of NAL and that the following replication time was normal. The experiment described in this section clearly demonstrated that chromosome replication in the cell cycle of R. palustris did not start until after 1.3 h incubation. This initial lag period, similar to that in Caulobacter (Degnen & Newton, 1972a), would not be expected in the development of mother cells in the second generation if DNA replication patterns of R. palustris and Caulobacter were truely similar. As in Caulobacter, it raises the problem of how initiation of chromosome replication might be controlled to occur at different times for the mother cell and swarmer. Diffusible cytoplasmic initiation proteins (Jacob et al, 1963) would be unlikely since initiator concentrations would be similar in both mother and daughter cells just prior to division. A structural or non-diffusible requirement which is only satisfied after a period of development such as swarmer maturation would seem a reasonable alternative.

Outgrowth of NAL-elongated cells after removal of NAL gave rise to three alternative patterns of cell division. Where complete or incomplete division resulted in two non-viable post-divisional cells this may have been due to irreversible denaturation of DNA before the NAL had been washed away from the treated synchronous culture. However, there were two other locations of division that occurred after removal of NAL which resulted in fully viable cells. Presumably the 1.5 h of continued outgrowth that was observed in both cases represented the time required for a complete round of chromosome replication to occur. This period of time correlates closely with the DNA synthesis period indicated by addition or removal of NAL at different developmental stages (p. 159 to 161). The observation of cell division occurring at either of two locations suggests that there were two possible positions for the unreplicated chromosomes to occupy in

elongated cells resulting from NAL treatment. This could be at the boundary between the normal tube and the bud swelling or at the tip of the bud swelling. Whichever location the chromosome occupied it must have remained membrane-bound in such a way to permit DNA replication and cell division to proceed on removal of NAL.

It has already been noted that the conventional model of DNA segregation during cell division (Jacob et al, 1963) cannot be directly applied to a budding organism such as R. palustris. A consideration of the regions of cell envelope growth (Fig. 92) and the observed cell division planes after reversal of NAL treatment (Fig. 91) allow a modification of the original model to be proposed. Such a model (Fig. 93) demands three new conditions to be fulfilled: (i) Each normal developing cell has two potential envelope growth points which occupy the same location up to bud formation and which then separate. One remains active at the pole of the cell and the other inactive at the end of the tube until division when it becomes duplicated. (ii) DNA-envelope association can only occur at potential growth sites. If the growth site becomes permanently inactive, attachment permanently ceases. (iii) Each potential envelope growth site can permit attachment of only one chromosome at a time. By this model chromosome segregation would normally occur as a result of bud outgrowth. During division of the cell the daughter chromosome attachment site and the envelope growth site become transferred from the cell tip to the division plane. When NAL inhibits DNA replication it presumably also inhibits duplication of the envelope attachment site. Only after removal of NAL could this duplication occur and thus the location of cell division is dependent on the location of the original single attachment site. By this means the observation of the two forms of cell division to give viable cells after the reversal of NAL treatment could be explained.

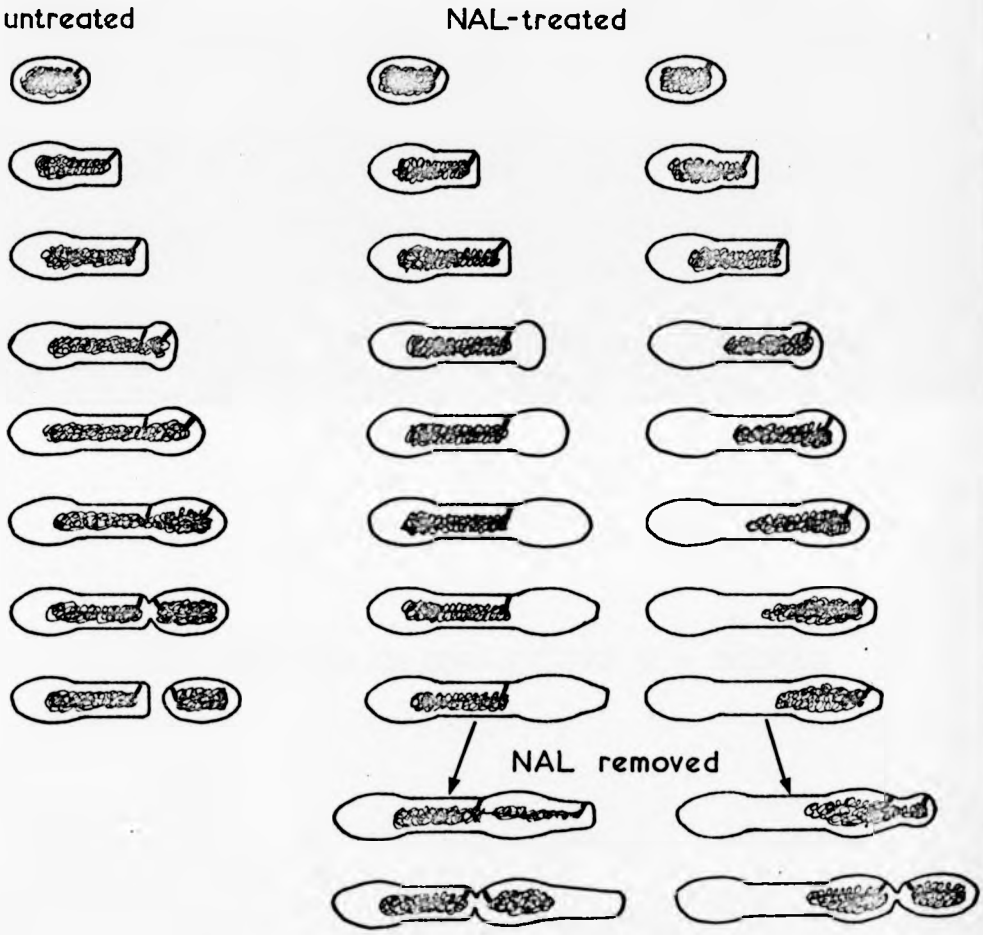


Fig. 93. Model of DNA segregation mechanism during cell cycle of R. palustris. Segregation is shown in the normal cell division cycle and in NAL-treated cells both before and after removal of the NAL.

SECTION III: MUTAGENESIS AND PHAGE INFECTION OF R. PALUSTRIS

SECTION III: MUTAGENESIS AND PHAGE INFECTION OF R. PALUSTRIS

INTRODUCTION

A number of different types of mutants of organisms to be used as models of bacterial differentiation are of benefit in studies of the cell cycle. Morphological and developmental mutants have direct application whilst nucleotide-requiring mutants may be useful for examining nucleic acid synthesis during the developmental cycle. Antibiotic-resistant mutants have most commonly been used to provide labelled organisms for strain recognition and examination of possible genetic transfer. To date no studies of morphological mutants or of genetic transfer in R. palustris strains have been reported in the literature. However, studies in other differentiation models have proved rewarding.

The facility of morphological mutants of an organism whose cell cycle is being studied is essential, not only to permit one to establish which phenomena are interrelated, but also to analyse the genes controlling the cellular functions. Such studies have been successful with B. subtilis sporulation (Ionesco et al, 1970; Hock, 1971; Brevet & Sonenshein, 1972) since the differentiation is not of an obligatory nature. Most developmental processes in Caulobacter are probably of an essential nature, but useful information has been gained on control of development using mutants which produce elongated stalks (Schmidt, 1968; Schmidt & Samuelson, 1972; Harrs & Schmidt, 1974). Because of the obligatory nature of R. palustris development one can envisage that many mutants would in fact inhibit growth or be lethal. Conditional mutants would permit the study of such mutations. Szulmajster, Bonamy & Laporte (1970) have studied the properties of a temperature-sensitive (t.s.) sporulation mutant of B. subtilis. Shapiro et al (1971) have also outlined some of the properties of t.s. mutants of C. crescentus in the context of a differentiation

model. Although these mutants were obtained by a method with no selective step (Hartwell, 1967) other selective methods are available, such as by killing growing cells at the restrictive temperature with penicillin.

Thymine is not incorporated into DNA by wild type E. coli and incorporation of thymidine rapidly stops, but continued incorporation of thymidine in the presence of ribo- and deoxyribonucleosides is possible (O'Donovan & Neuhard, 1970). Preliminary experiments with R. palustris showed no incorporation of thymidine at all in the presence of uridine, whilst Dow (1974) could only obtain incorporation of thymidine by Rm. vannielii for 1 to 2 h with or without uridine. If thymine-requiring (thy$^-$) mutants of R. palustris could be obtained such problems may be overcome and an accurate and specific assay of DNA replication could be possible as well as permitting experiments on thymine starvation. Stacey & Simson (1965) have described a selective method of obtaining thy$^-$ mutants, this method later having been modified by Andrews (1973) for selection of thy$^-$ Streptococcus faecalis isolates.

Mutants of B. subtilis which were selected as being resistant to rifampicin have also been found to exhibit abnormal sporulation or no sporulation at all (Doi et al, 1970; Sonenshein & Losick, 1970). This seems to be due to formation of an altered DNA-dependent RNA polymerase (the target of rifampicin) whose subunit structure cannot be modified in the normal way for transcriptional control of sporulation, but continues to transcribe normal vegetative cell material (Sonenshein & Losick, 1970; Losick, Shorenstein & Sonenshein, 1970). Bendis & Shapiro (1973) compared RNA polymerase from C. crescentus stalked cell populations with swarmer cell-enriched or pre-divisional cell-enriched populations, but the enzyme was apparently the same in all samples. If rifampicin-resistant mutants of R. palustris could be obtained it may be possible to say whether changes in RNA polymerase are involved in developmental control in this organism.

Resistance to rifampicin as well as to other antibiotics might offer particularly useful genetic markers. Not only would these allow easy recognition of genetic transfer but they would also permit easy recognition of mutants as being from the mutagenised culture and not contaminants. For any proposed model of differentiation it is desirable that a mechanism of genetic exchange should be available. This would permit one to (i) establish that apparent t.s. mutants are as a result of genetic alterations, (ii) investigate genetic aspects of altered development, and (iii) ultimately map the regulatory gene loci concerned with differentiation (Shapiro et al, 1971). Genetic transfer in budding bacteria has not yet been reported in the literature. Zavarin (1961) suggested that in Hyphomicrobium, during aggregation of cells by their poles, conjugation might occur, but there is no evidence of this occurring. Schmidt (1971) pointed out that Caulobacter possesses RNA phage-specific pili and that this might indicate a capability for conjugation. Indeed, Shapiro et al (1971) presented information indicating that genetic exchange of this type does occur.

Although transformation only occurs under highly specific conditions (Notani & Setlow, 1974), genetic transfer by transduction has been easily achieved using temperate phages of a number of bacteria. Transduction using a virulent phage has now also been achieved in E. coli where amber phage mutants could infect but not multiply in non-permissive recipients (Drexler, 1970). Only one phage has been reported which infects R. palustris (Freund-Mölbert et al, 1968); however this has been found to be specific for only one previously reported host strain. Unless other R. palustris-specific phages can be isolated attempts at transduction would have to be confined to this one phage and its host.

Phages have a number of other uses in studies of developmental cycles. Phage receptor sites have been recognised as occuring at specific

locations during development. Voelz, Gerencser & Kaplan (1971) demonstrated the absorption of a phage, specific for a Hyphomicrobium sp. (Gerencser & Voelz, 1971), at the growing tip of the filament and bud. Similar results were observed by Bosecker, Drews & Tauschel (1972) when examining the adsorption of phage Rp1 to its R. palustris host cells. It is important to note that their experiments were not carried out using synchronous cultures and, in addition, their summary of the developmental cycle of R. palustris was incorrect. They described symmetrical, equal division producing two similar progeny cells rather than a tubed mother cell and a non-tubed daughter cell (see Sections I and II of this thesis).

Shapiro & Agabian-Keshishian (1970) used an RNA phage which adsorbs onto pili as a tool for specifically assaying development in synchronous cultures of Caulobacter. As swarmers matured they lost their flagella and pili and consequently the proportion of adsorbing phage dropped. Complementary to this assay, Shapiro et al (1971) have described the use of a DNA phage, which only adsorbs during the swarmer synthesis on the stalked cell, as a further assay of development. Clearly, phages which are specific for receptor sites which appear periodically or at changing locations during development of an organism are useful for recognising the occurrence of the receptor sites as well as being used as specific assays of development.

In this section are described some preliminary experiments carried out to investigate whether the mutants necessary for elucidating developmental processes and their control could easily be obtained in R. palustris. In addition, the possibility of using conjugation or transduction for genetic transfer was examined.

MATERIALS AND METHODS

Bacterial strains and their cultivation. R. palustris strain C1 and R. palustris strain 1e5 were obtained from the sources described in Section I (p.29). E. coli strain AB1157 and its recA⁻ counterpart, strain AB2463, were obtained from Dr S.B. Primrose (University of Warwick, Coventry).

ATCC medium No. 550 consisted of a complex malic acid-salts-yeast extract-trace elements-vitamins combination of nutrients as described in the ATCC Catalogue of Strains (10th Edition, 1972). PAYE medium for R. palustris was prepared as described in Section IIA (p. 61); PA medium consisted of the same nutrients as PAYE but without yeast extract. Procedures for aerobic and anaerobic culture of photosynthetic bacteria have already been described (Section I, p. 30) as has the anaerobic bag technique for photosynthetic incubation of agar plates (p. 32).

Spectrophotometric measurements of cells were made using a Pye-Unicam SP500 spectrophotometer, bacterial cultures being grown for this purpose by the cuvette culture method described in section IIB (p. 95).

Light microscopy of bacteria was usually carried out on an Olympus EHT microscope fitted with a PM-6 camera. Electron microscopy was carried out on an AEI Corinth 275 at an accelerating voltage of 60 KV and electronmicrographs taken on Ilford 70 mm line film, N4E50. Negative staining of preparations was carried out using 1% potassium phosphotungstate, pH 7.0.

Chemicals and buffers. N-methyl-N'-nitro-N-nitrosoguanidine (NTG; obtained from Ralph N. Emanuel Ltd, Wembley) was prepared as a 1 mg/ml aqueous solution by shaking at room temperature until dissolved. 2,4-diamino-5-(3,4,5-trimethoxy benzyl)-pyrimidine (trimethoprim) was obtained from Sigma (Sigma Chemical Co., London) and prepared as an

aqueous solution. Rifampicin, chloramphenicol and nalidixic acid (NAL) were all obtained from Sigma and prepared as solutions as described in Section IIC (p. 130). Thymine (Sigma) was dissolved in water with warming as necessary. If required, all the above chemicals were sterilised by filtration through Millipore 0.22 μm GS filters.

Tris-maleic buffer (0.05 M) consisted of 6.1 g/l of tris-(hydroxymethyl)-methylamine and 5.8 g/l maleic acid, adjusted to pH 6.0 with NaOH.

0.1 M TES buffer was prepared containing tris-(hydroxymethyl)-methylamine, 12.11 g/l; NaCl, 5.845 g/l; EDTA, 3.73 g/l, and adjusted to pH 7.1.

Phage buffer (0.01 M) was prepared as a ten-times concentrate containing tris-(hydroxymethyl)-aminomethane, 12.1 g/l; $MgCl_2.7H_2O$, 10.15 g/l; $CaCl_2.2H_2O$, 1.11 g/l (Freund-Möbert et al, 1968).

Bacterial mutagenesis. Cells were treated with freshly generated nitrous acid by mixing 5 ml cell suspension with 5 ml sterile 0.05 M $NaNO_2$ and 5 ml of sterile 3 M acetate buffer, pH4.5 (100 ml 3 M acetic acid + 43 ml 3 M NaOH), added to start the mutagenesis (Kaudewitz, 1959). The culture was incubated anaerobically with illumination at 30° and, when desired, mutagenesis in samples stopped by an initial dilution of 1/100 into phosphate buffer.

For mutagenesis with NTG cells were first resuspended in tris-maleic buffer and then 9 ml of cell suspension treated with 1 ml of NTG to give a final concentration of 100 μg/ml (Adelberg, Mandel & Chen, 1965). During mutagenesis the cell suspension was incubated under illumination at 30°. Treatment with NTG was stopped by 100-fold dilution of samples into phosphate buffer.

Selection of t.s. mutants. A number of possible t.s. mutants of R. palustris C1 were kindly obtained by Dr S.B. Primrose: a culture of R. palustris C1, resistant to both nalidixic acid and chloramphenicol,

was mutagenised with NTG, grown up photosynthetically at 30° until turbid and then sub-cultured (0.5% inoculum) at 42° and grown for a further 8 h. Penicillin was then added to the culture to a concentration of 4,000 IU/ml and the culture incubated photosynthetically for a further 16 h. Surviving bacteria were plated out and grown up at 30° using an anaerobic bag. All isolates which grew at 30° on PAYE plates but not at 42° were selected as presumptive t.s. mutants.

Selection of antibiotic-resistant mutants. After mutagenesis of cultures with NTG, mutants resistant to rifampicin, chloramphenicol, NAL and oxytetracycline were selected as follows. Cells were spread on PAYE agar plates and in the centre of each plate was placed a filter paper disc containing antibiotic. For rifampicin, 2 cm Whatmans 3MM filter paper discs were used containing approximately 100 μg antibiotic. For chloramphenicol, NAL and oxytetracycline, Oxoid sensitivity discs (Oxoid Ltd, London) were used which contained 50 μg, 30 μg and 50 μg, respectively. After photosynthetic incubation of the plates isolated colonies of resistant organisms were picked off from the zones of clearing and sub-cultured in antibiotic-containing medium.

Extraction and ultracentrifugation of bacterial DNA. About 1 l of late exponential culture was centrifuged, washed and resuspended in about 50 ml 0.1 M TES buffer (pH 7.1). The pre-warmed suspension of cells was treated with lysozyme (Sigma; 4 mg/ml final concentration) for 1 h at 37°. Lysis of cells was completed by dropwise addition of a 5% solution of sodium lauryl sarcosinate until the suspension became highly viscous. DNA was separated from cellular debris, RNA and protein by centrifugation to equilibrium at 10° in CsCl of mean density of 1.71 g/cm^3 for 36 h at 120,000 x g. (MSE Super Speed 65 centrifuge). The DNA was detected as a viscous fraction part way down the gradient and collected via a hole pierced through the bottom of the centrifuge tube. The extracted DNA was

dialysed against 0.1xSSC (10-times concentrate contained 87.7 g/l NaCl and 44.2 g/l sodium citrate) and sample concentration and purity then checked by measurement of its A_{260} and the ratio A_{280}/A_{260}.

DNA from Micrococcus lysodeikticus was obtained as a highly polymerised dehydrated material from Sigma, and used as DNA of standard buoyant density.

Analytical ultracentrifugation was carried out in a Beckman Model E analytical ultracentrifuge. A solution of CsCl (density of 1.71 g/cm^3) was prepared with 0.1 M TES buffer, pH 7.1, containing 1 to 2 µg of each species of DNA to be tested, and was loaded into the 12 mm 4° Kel F centrepiece of the centrifuge cell. Centrifugation of the samples was for 22 h at 44,000 rev/min and at 25°. Photographs were taken using u.v. absorption optics and negatives reproduced as prints and microdensitometer scans (Chromoscan; Joyce Loeble & Co. Ltd, Gateshead). The buoyant densities and guanine plus cytosine ratios were calculated by comparison with M. lysodeikticus (1.7310 g/cm^3; 72.45% G+C) using the method of Mandel, Shildkraut & Marmur (1968).

Phages and plaque assays. Attempts to enrich and isolate phages which cause lysis of R. palustris C1 from freshwater and sewage samples were unsuccessful. To allow some studies to be pursued the phage Rp 1, specific for R. palustris 1e5, was obtained from the American Type Culture Collection (ATCC 25852B).

Plaque assaying of the phage was carried out by the following method. A 0.1 ml sample of diluted phage suspension was mixed with 2 ml of PAYE agar which contained only 0.7% agar, maintained at 55°, and 0.2 to 0.5 ml of late exponential host cell suspension added to the mixture. This soft agar-phage-cell mixture was then overlayed onto PAYE agar in Petri dishes, allowed to set and then incubated photosynthetically in an anaerobic bag at 30° for 1 to 5 d.

Phage mutagenesis. Nitrous acid mutagenesis (Snustad & Dean, 1971) of Rp 1 phage was carried out by mixing 0.9 ml of phage suspension, 0.1 ml of 40 mg/ml $NaNO_2$ and 1.0 ml of 0.1 M acetate buffer, pH 5.0 (36 ml 0.1 M sodium acetate + 64 ml 0.1 M acetic acid). The mixture was incubated at room temperature and mutagenesis of samples stopped when required by diluting 1/100 in PAYE.

Mutagenesis of Rp 1 phage was also attempted by u.v. irradiation. 2.5 ml of phage suspension was placed in a sterile 6 cm glass Petri dish together with a sterile slave magnet. The u.v. lamp was allowed to warm up for half an hour after which time 1.2 x 10^4 ergs/sec/cm^2 irradiation was detected at a distance of 15 cm. Irradiation of the stirred phage suspension was started by removing the Petri dish lid and mutagenesis of samples stopped as they were drawn up into the glass pipettes.

Centrifugation of phage in CsCl. A bulk preparation of phage-containing lysate was made using preparative soft agar plates. 45 plates were prepared, overlaying with 2.0 ml soft PAYE agar (0.7% agar), 2.0 ml cell suspension and 0.1 ml of phage dilution calculated to give confluent lysis of the cells (about 10^4 p.f.u./ml in this case). After photosynthetic incubation of the plates at 30°for 2 d the soft agar was pooled and liquidised with an equal volume of phage buffer by vigorously stirring for 2 to 3 h. The agar was removed by centrifugation at 10° twice for 20 min at 10,000 x g. (MSE High Speed 18 centrifuge). Aliquots of the phage-containing supernate were loaded on the top of 12 ml quantities of saturated CsCl solution in 65 ml centrifuge tubes and centrifuged at 10° for 2.5 h at 45,000 x g. (MSE Super Speed 65 centrifuge). Band-containing material in the resulting equilibrium gradients was pooled and dialysed against phage buffer. The dialysed material was then loaded onto 18 ml quantities of saturated CsCl in 65 ml centrifuge tubes and re-centrifuged at 10° for 2.5 h at 45,000 x g. The resulting bands, about half way down

the equilibrium gradient, were then dialysed against phage buffer before examining under the electron microscope.

RESULTS

Mutagenesis of R. palustris

In order to find a suitable mutagen for R. palustris C1 and the required time of treatment, survival of cultures after mutagenesis was investigated. A u.v. lamp that was available was tested for its bactericidal action. At 20 cm distance a u.v. sensitive recA⁻ mutant of E. coli (strain AB2463) gave 0.1% survival after 1 min exposure; however, a normally sensitive E. coli (strain AB1157) gave more than 10% survival after 3.5 min exposure. Clearly the lamp was giving an insufficiently high u.v. emission for conveniently obtaining mutants.

Chemical mutagenesis of R. palustris was investigated using nitrous acid or NTG. In order to avoid clumping of cells and rosette formation interfering with cell viable counts a semi-synchronised (about 80% swarmers) culture was used. 0.017 M nitrous acid was generated as described in Materials and Methods in a portion of the culture, cell samples being taken before treatment and after 0, 10, 20, 30 and 40 min anaerobic incubation with nitrous acid. Serial dilutions of each sample were counted after growth of survivors on spread plates incubated photosynthetically. Fig. 94 shows that under these conditions there were less than 1 in 2.5×10^6 survivors after 10 min treatment. For a low level of treatment such as used in this method the level of kill observed was exceptionally high. However, it was noticed that the organism turned a pale green colour, perhaps indicating some chemical action of nitrous acid on photopigments of R. palustris.

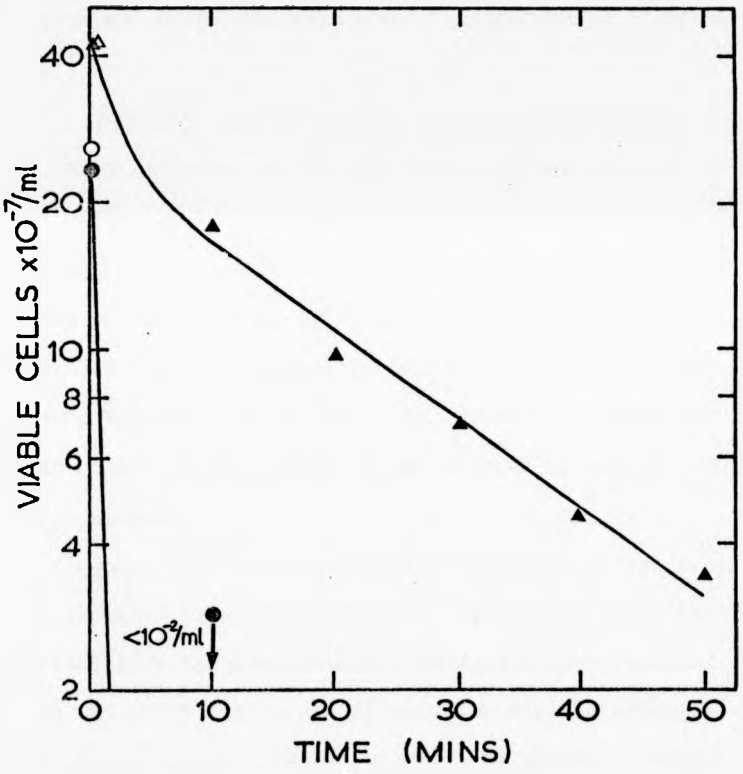


Fig. 94. Survival curves of R. palustris C1 after chemical mutagenesis. Numbers of surviving viable bacteria were estimated before (open symbols) and during (closed symbols) mutagenesis with freshly generated 0.017 M nitrous acid (○,●) or 100 µg/ml NTG (△,▲).

Cells resuspended in tris-maleic buffer were treated with NTG to give a final concentration of 100 μg/ml. Again, cell samples were taken before treatment and after 0, 10, 20, 30, 40 and 50 min anaerobic incubation with NTG. Plate counts of serial dilutions gave the logarithmic death plot shown in Fig. 94. It was clear that after about 45 min treatment with 100 μg/ml NTG there was 10% survival. This should yield large numbers of mutants.

Screening for non-lethal morphological mutants

Since mutants exhibiting altered morphology at a restrictive temperature might yield some clues as to control of development, a population of R. palustris C1 was mutagenised with 100 μg/ml NTG for 45 min and spread plates of the survivors grown up at 37° aerobically in the dark. It was anticipated that abnormal morphology might be revealed in altered colony form, allowing selection to be made. In addition, aerobic dark incubation would permit growth of any mutants whose altered morphology would not permit photosynthesis.

Amongst the numerous normally appearing colonies were a number with slightly altered morphology. 95 such colonies were sub-cultured at 37°aerobically in the dark on agar plates and also examined microscopically. Two isolates (Rp NTG70 and Rp NTG76) revealed curious spherical cell bodies separated by a much narrower tube. Isolate Rp NTG76 was examined first. Fig. 95 shows the appearance of the organism at 37° grown aerobically in the dark. Because of the length of the tube separating the pairs of spherical cells and the fact that it gave a Gram negative reaction it seemed unlikely that the isolate was a contaminating diplococcus. However, cell dimensions were considerably greater than the wild type R. palustris C1. Unfortunately, after a few sub-cultures only normally appearing R. palustris cells remained. There had either been a reversion to wild type or death of the mutant isolate. As a result of this no adequate studies on growth at

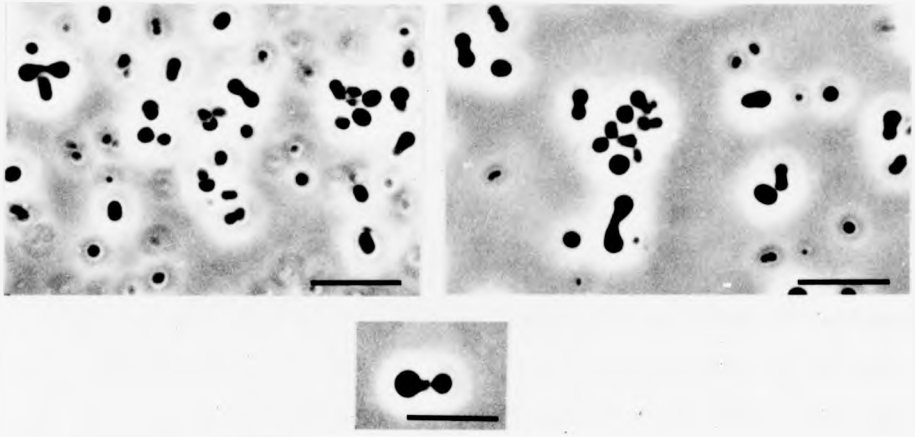


Fig. 95. Morphological mutant of R. palustris C1 (Rp MTG76). Bars represent 10 µm.

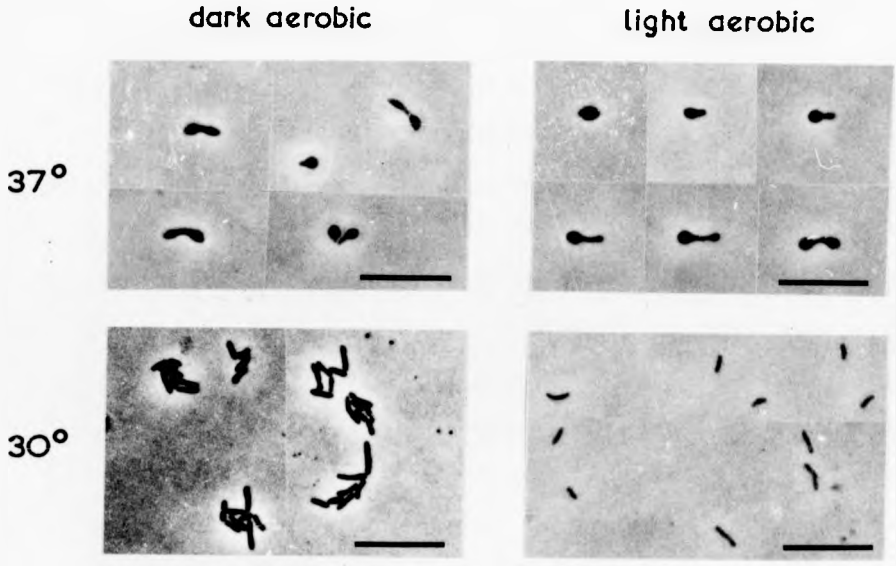


Fig. 96a-d. Temperature-sensitive morphological mutant of R. palustris C1 (Rp MTG70). Bars represent 10 µm.

other temperatures and under different environmental conditions could be undertaken.

Rather more was established about isolate Rp NTG70. The initial colony, grown at 37° aerobically in the dark, was small and non-pigmented. However, microscopic examination revealed swollen cells remeniscent of Rp NTG76, although of a size similar to wild type R. palustris. The isolate was then sub-cultured in liquid medium under a number of different conditions and examined microscopically. Aerobic dark growth at 37° was scanty and non-pigmented and again revealed terminal swellings linked by a narrower tube (Fig. 96a). Little growth was observed at 37° aerobically in the light but the few cells that were seen appeared the same as in the dark (Fig. 96b). Aerobic dark growth at 30° gave rise to a good density of apparently non-pigmented cells. Most cells appeared to have a similar morphology to wild type R. palustris (Fig. 96c) also being motile and occasionally sticking to the coverslip by one cell pole. The most surprising observation was made at 30° when cultures were incubated aerobically in the light. Under these conditions, although the cells appeared the same as under dark incubation at 30° (Fig. 96d), the culture was a pale yellow colour. When cells from this type of culture were suspended in saturated sucrose and their whole cell absorption spectrum compared with that of cells grown aerobically in the dark at 30° it was clear that there was a broad pigment peak at about 450 nm (Fig. 97). Negligible growth of Rp NTG70 was detected under anaerobic illuminated conditions indicating that although it was capable of some light-induced pigment synthesis it was not capable of photosynthesis.

Again, problems were faced with maintaining cultures of the isolate. This time it was noticed that after some sub-cultures aerobic dark cultures were growing with a yellow pigmentation. Microscopic observations revealed that most cells which grew at 30° were non-motile,

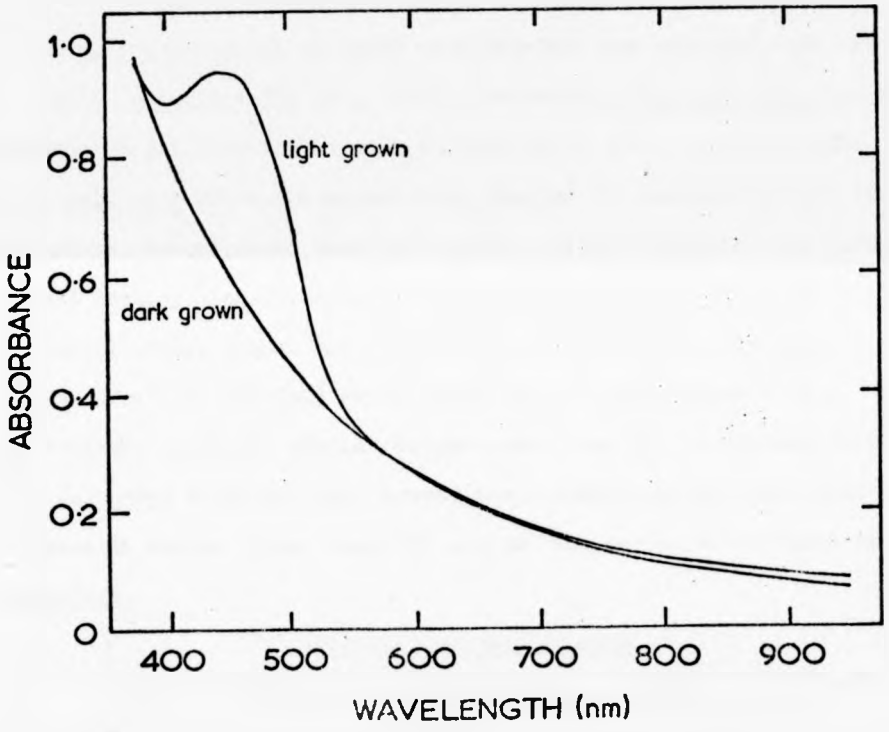


Fig. 97. Whole cell absorption spectra of isolate Rp NTG70. The isolate was grown aerobically at 30° in the light or in the dark and cell spectra obtained from cells suspended in saturated sucrose solution.

did not form rosettes and did not produce terminal buds. However, at 37° a number of cells with terminal swellings were still seen. Since there was some suspicion that a yellow contaminant growing only at 30° had overtaken the growth of isolate Rp NTG70 a buoyant density analysis was carried out on DNA extracted from a dark grown aerobic culture at 30°. DNA from M. lysodeikticus was used as a standard and compared with extracted wild type R. palustris DNA (Fig. 98a). Secondly, M. lysodeikticus DNA was compared with DNA from the unknown culture (Fig. 98b). Finally, wild type R. palustris DNA was compared with that of the unknown culture (Fig.98c). This analysis demonstrated that the unknown culture contained DNA having a buoyant density almost identical to that of M. lysodeikticus (ρ=1.7310 g/cm^3; 72.45% G+C); rather higher than that of the wild type R. palustris (ρ=1.7238 g/cm^3; 65.05% G+C) which was clearly distinguishable as a separate peak. Although similar buoyant densities of the unknown culture and R. palustris would not have proved their common origin, the observed difference in values proved that the unknown was no longer a mutant of R. palustris.

Antibiotic-resistant mutants

Rifampicin-resistant (rif^R) mutants were sought in the hope that they might exhibit some morphological modifications and abnormalities. The mutants were selected from an NTG mutagenised population of R. palustris C1 as described in Materials and Methods. 15 colonies growing in zones of clearing of the lawns of cells around filter paper discs containing rifampicin were picked off and grown up photosynthetically in PAYE medium containing 40 µg/ml rifampicin. Wild type R. palustris C1 did not grow in 3 d under these conditions although all 15 isolates did. Only after 6 d was there slight turbidity in the wild type culture, indicating the growth of spontaneous mutants. Microscopic observations of all the isolated rif^R cultures revealed no obvious developmental abnormalities or

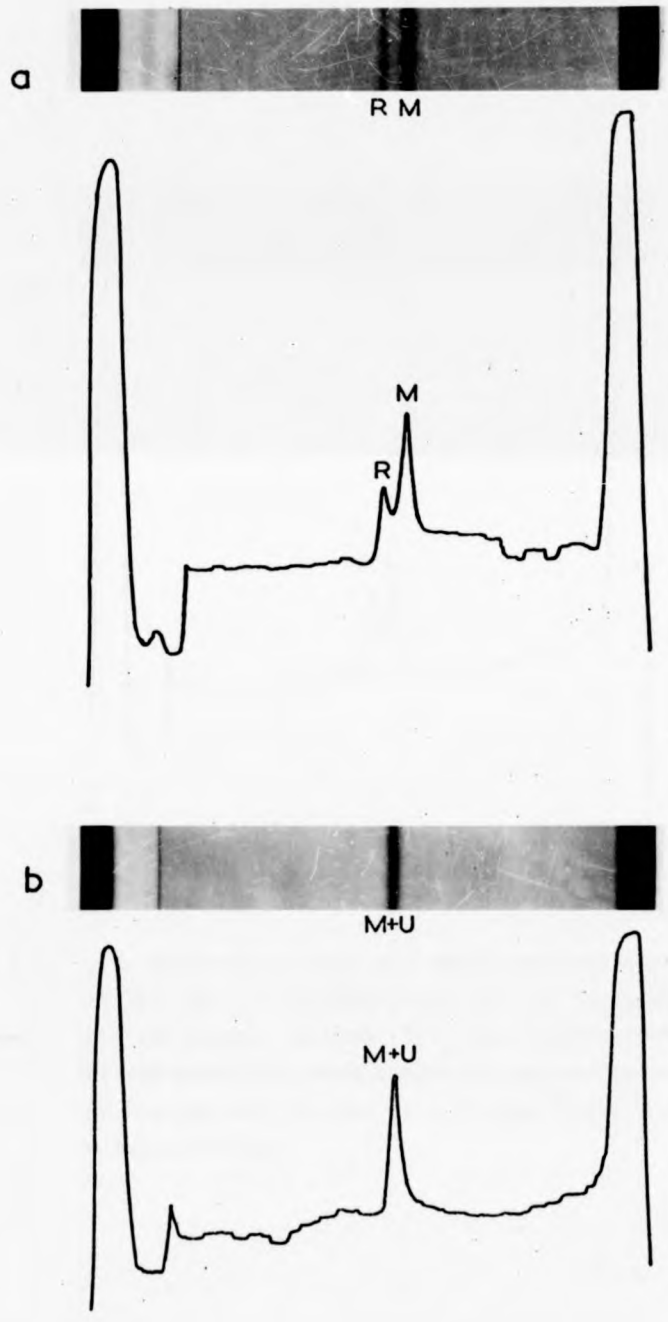


Fig. 98a & b. See page 194 for title and legend.

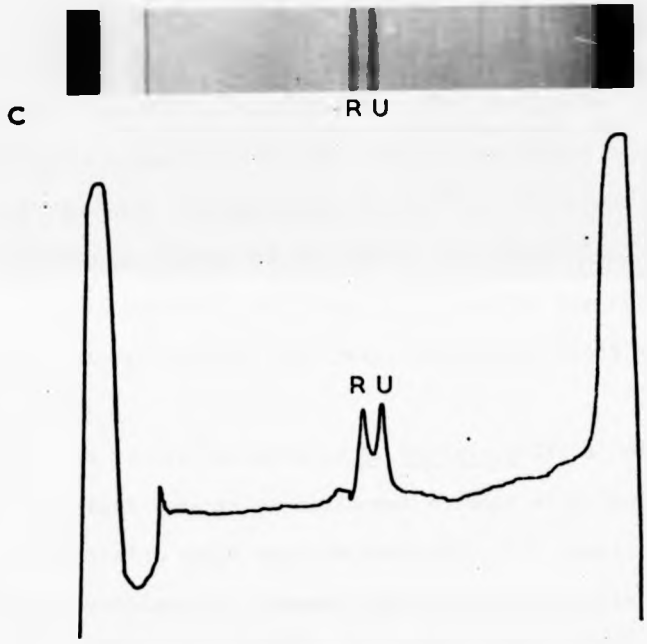


Fig. 98a,b & c. u.v. absorption bands and resulting densitometer scans of DNA from M. lysodeikticus (M), R. palustris C1 (R) and an unknown culture (U). The various combinations of extracted DNA were banded by centrifugation for 20 h at 44,000 rev/min on a Beckman Model E analytical ultracentrifuge.

modifications. However, it was desirable to determine to what extent these mutants were resistant to rifampicin so that suitable concentrations of antibiotic could be used to permit growth of the isolates and also to characterise the mutants. One loopful of each of the 15 rif^R mutants and wild type R. palustris C1 were sub-cultured into 5 ml aliquots of PAYE containing 0, 50, 100, 150, 200 and 250 µg/ml rifampicin, each treatment being carried out in duplicate. Cultures were incubated photosynthetically and examined for growth after 3 d and 6 d. Table 6 indicates the amount of growth that was observed. R. palustris C1 rif^R1, 2, 3, 4 and 14 exhibited an extremely high degree of resistance to rifampicin. However, their morphology was not obviously different from that of the wild type indicating that such mutations would not give information about transcriptional control of development.

In addition to rif^R mutants of R. palustris C1, a number of other antibiotic-resistant mutants of different strains of R. palustris were sought. These isolates would then be available with genetic markers for genetic transfer experiments. Several chloramphenicol-resistant and NAL-resistant mutants were easily obtained and also a few oxytetracycline-resistant mutants. It would appear that a variety of antibiotic-resistant mutants of R. palustris can easily be obtained.

Temperature-sensitive mutants of R. palustris

A mutant of R. palustris C1 which was both NAL-resistant (nal^R) and chloramphenicol-resistant (cm^R) was used in this t.s. mutant selection to provide a means of recognising isolates as being mutants and not contaminants. Eight mutants were obtained which gave good growth on photosynthetically incubated agar plates at 30° but only poor or negligible growth at 42°. The growth properties of the isolates were tested in liquid media. One loopful of cell suspension of each isolate was used to inoculate 10 ml aliquots of PAYE containing 50 µg/ml NAL and 50 µg/ml

Table 6. Growth of *R. palustris* C1 wild type and rif^R mutants in the presence of various concentrations of rifampicin.

	growth after 3 d						growth after 6 d					
isolate	0*	50	100	150	200	250	0	50	100	150	200	250
wild type	+	-	-	-	-	-	++	-	±	-	-	-
rif^R 1	+	+	+	+	+	+	++	++	++	++	++	++
rif^R 2	++	++	+	+	+	+	++	++	++	++	++	++
rif^R 3	++	+	+	+	+	+	++	++	++	++	++	++
rif^R 4	++	++	++	+	+	+	++	++	++	++	++	++
rif^R 5	++	+	+	-	-	-	++	++	++	+	±	-
rif^R 6	++	++	+	-	-	-	++	++	++	+	±	-
rif^R 7	++	+	-	-	-	-	++	++	++	++	++	++
rif^R 8	+	+	+	-	-	-	++	++	++	++	+	+
rif^R 9	++	+	±	-	-	-	++	++	++	++	++	++
rif^R 10	++	++	+	±	-	-	++	++	++	++	++	++
rif^R 11	+	+	±	-	-	-	++	++	++	+	±	-
rif^R 12	++	+	±	-	-	-	++	++	++	++	+	+
rif^R 13	++	+	+	-	-	-	++	++	++	++	+	+
rif^R 14	++	++	++	+	+	+	++	++	++	++	++	++
rif^R 15	+	+	+	±	-	-	++	++	++	++	++	++

*, μg/ml rifampicin

-, no growth

±, negligible growth

+ to ++, arbitrary increasing scale of growth

chloramphenicol. For each isolate one bottle was incubated at 30° and one at 42°. After 3 d photosynthetic incubation growth was compared with similar cultures of wild type and R. palustris nal^R cm^R and also with these two controls grown in the absence of antibiotics. Table 7 summarises the observed growth. Although all the t.s. isolates were only able to grow at 30°, so was the nal^R cm^R parent strain. This was not due to the inability of R. palustris C1 to grow at 42° as growth was observed in the control cultures without antibiotics at both temperatures. It appeared that the nal^R cm^R properties of the parent culture had in fact been t.s.. This was checked by growing the nal^R cm^R R. palustris at 30° or 42° in the presence of both antibiotics or NAL or chloramphenicol separately (Table 8). Growth at 42° was only observed in culture medium containing no chloramphenicol. The parent culture was, therefore, nal^R cm^{ts} R. palustris.

In order to determine whether there were any additional t.s. properties of the t.s. isolates, growth at 30°and 42° was compared in media containing no antibiotic (Table 9). Under these conditions little or no growth was observed in isolates ts8, ts9, ts13 and ts14, whereas the other isolates and the nal^R cm^{ts} parent strain grew at least as well as the wild type at 42°. Thus it would appear that these four isolates possessed additional t.s. mutations. So that it could be determined whether any of the isolates had a t.s. block in the cell cycle cultures were grown in PAYE (no antibiotics) photosynthetically at 30° for 24 h until turbid and then the incubation temperature increased to 42°. After a further 16 h incubation morphology of the cultures was examined. Fig. 99 shows the appearance of such cultures: none had stopped developing at a single stage of the developmental sequence in the cell cycle. Isolate ts8 contained few swarmer cells but a number of cells showed incomplete division and Y-forms of dividing cells were also observed. Isolate ts9 contained rather narrow cells of all lengths including swarmers. Dividing cells and

Table 7. Growth of *R. palustris* C1 wild type, nal^R cm^R parent strain and t.s. isolates at 30° and 42° in PAYE plus 50 µg/ml NAL and 50 µg/ml chloramphenicol (CM).

	PAYE		PAYE + NAL + CM									
temp.	wt	$nal^R cm^R$	wt	$nal^R cm^R$	ts1	ts 2	ts3	ts5	ts8	ts9	ts13	ts14
30°	++	+++	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
42°	++	++	-	-	-	-	-	-	-	-	-	-

Table 8. Growth of *R. palustris* C1 $nal^R cm^R$ at 30° and 42° in PAYE containing 50 µg/ml NAL or 50 µg/ml CM or both antibiotics.

	PAYE + NAL + CM		PAYE +NAL		PAYE + CM	
temp.	3 d	4 d	3 d	4 d	3 d	4 d
30°	+	+++	+++	+++	++	+++
42°	±	±	++++	++++	±	±

Table 9. Growth of *R. palustris* C1 wild type, nal^R cm^R parent strain and t.s. isolates at 30° and 42° in PAYE.

	PAYE									
temp.	wt	$nal^R cm^{ts}$	ts1	ts2	ts3	ts5	ts8	ts9	ts13	ts14
30°	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
42°	+	++	+	+	++	+++	±	±	-	±

For Tables 7, 8 & 9: wt, wild type
-, no growth
±, negligible growth
+ to ++++, arbitrary increasing scale of growth

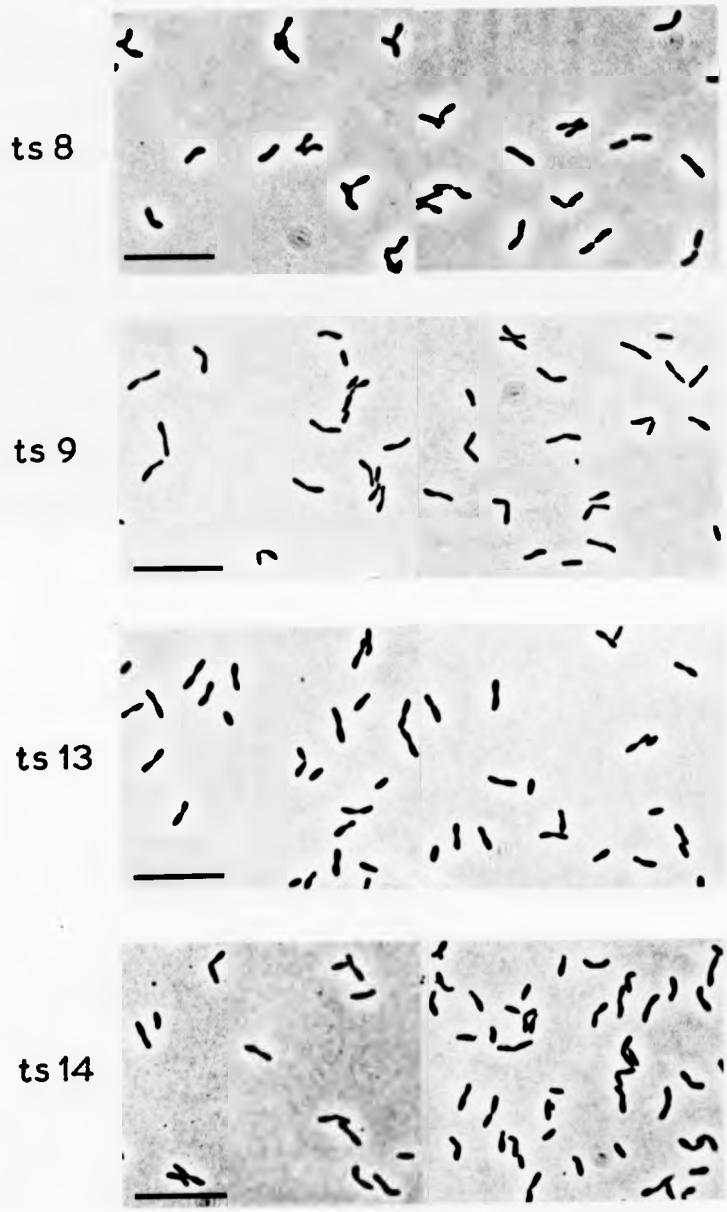


Fig. 99. Appearance of t.s. mutants of R. palustris C1 at the restrictive temperature (42°). Bars represent 10 µm.

cells with well developed buds were uncommon and cell tubes often appeared elongated. The morphology of isolate ts13 was quite normal and exhibited cells of all kinds. Isolate ts14 also exhibited cells of all types, however there were a number of "bent" cells and a few with Y-forms, perhaps suggesting a similarity to ts8.

Since a single developmental stage was not observed in any culture at the restrictive temperature investigations of the t.s. isolates were not continued. However, these experiments did demonstrate the fact that t.s. mutants of R.palustris C1 may easily be obtained and perhaps screening of larger numbers of isolates may yield several useful t.s. mutants. It is also possible that further investigation of isolates ts8 and ts9 might give information on the processes of cell division and elongation in R. palustris.

Selection of thymine-requiring mutants of R. palustris

Since adequate levels of incorporation of radioactive thymine and thymidine into DNA of R. palustris C1 had not been possible, a thy⁻ mutant was sought which would then permit such incorporation. Experiments were carried out using trimethoprim as an agent favouring the growth of thy⁻ strains. PA medium was prepared containing 1 mg/ml p-aminobenzoic acid and used as a minimal medium for thy⁻ selection by the procedure of Stacey & Simson (1965). Isolates were then tested by replica plating on medium with and without thymine. Every tested isolate grew well on both plates. This result would suggest that R. palustris C1 might be impermeable or insensitive to trimethoprim. There was certainly very little inhibition of growth of the wild type R. palustris by trimethoprim and further investigations showed that wild type cultures were able to produce lawn growth right up to crystals of trimethoprim placed on spread plates of R. palustris.

Selection of thy⁻ mutants was also attempted by the modified method of Andrews (1973). A culture of R. palustris was grown in defined

medium supplemented with 10 μg/ml trimethoprim until stationary phase of growth was reached (24 h). These cells were then used as inoculum for medium containing 10 μg/ml trimethoprim and 50 μg/ml thymine. After 5 h photosynthetic growth surviving bacteria were cloned and tested for thymine requirement by replica plating. Again no thy⁻ isolates were obtained.

Purification and some properties of R. palustris strain 1e5

Since R. palustris strain 1e5 has been reported to be the only host strain for the phage Rp 1, a freeze-dried vial of the culture was obtained from ATCC and grown up for examination. A portion of the freeze-dried material, resuspended in ATCC medium No. 550, revealed a mixed culture containing equal proportions of a R. palustris-like organism and a spirillum (Fig. 100). Clearly the ATCC stock cultures were grossly contaminated. Agar plates of ATCC medium No. 550 streaked with the mixed culture and incubated photosynthetically for 4 d at 30° gave rise to small red colonies typical of R. palustris and large bright red colonies of cells closely resembling Rhodospirillum rubrum. It was established that pure cultures of R. palustris strain 1e5 grew satisfactorally in PAYE medium and, this being a considerably simpler medium, was used in the remaining studies.

Although cultures of R. palustris 1e5 were initially motile, this property was largely lost upon sub-culture. When compared with the strain of R. palustris used in the cell cycle studies in this thesis (R. palustris C1), strain 1e5 was seen to have a tendancy to produce curved swarmer cells and hence also curved mother cells (Fig. 101). Although it was not so obvious in photographs, direct examination of cells by phase contrast microscopy clearly revealed a phase contrast-translucent tube between mother and daughter cells, though short and often tapering. The fact that R. palustris 1e5 responds to phosphate-limited growth has already been described in Section I of this thesis (p. 43).

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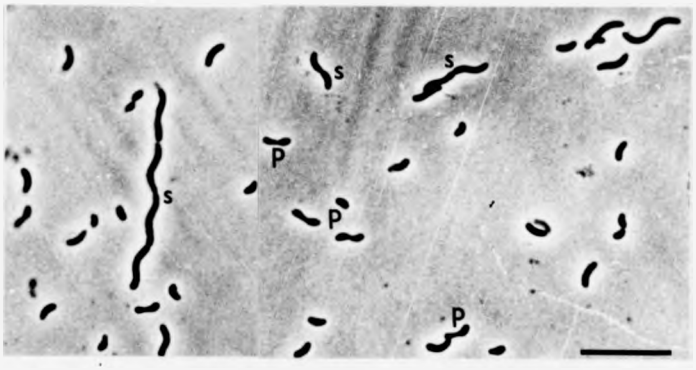


Fig. 100. Rehydrated contents of ATCC vial of freeze-dried R. palustris 1e5. The cell suspension contained R. palustris-like cells (P) as well as a spirillum (S). Bar represents 10 µm.

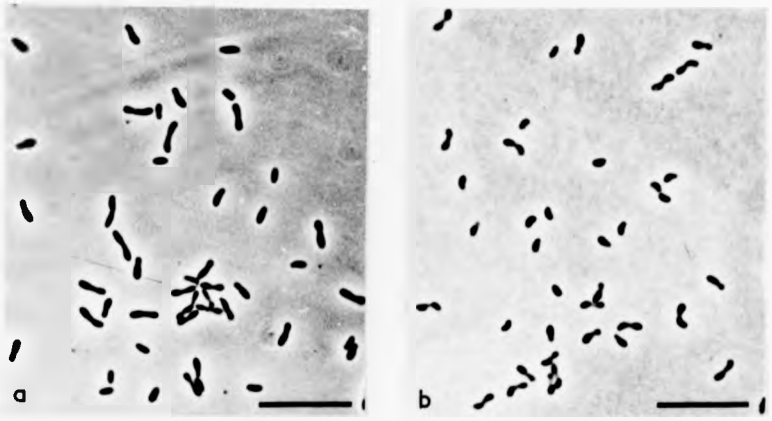


Fig. 101. Pure cultures of R. palustris C1 (a) and R. palustris 1e5 (b). Bars represent 10 µm.

A further comparison of R. palustris C1 and 1e5 was made by extracting DNA of both isolates and analysing their buoyant densities. Comparison of M. lysodeikticus and R. palustris C1 (Fig. 102a) indicated that on this occasion the latter had a buoyant density of 1.7233 g/cm^3 (64.64% G+C). Comparison of M. lysodeikticus and R. palustris 1e5 (Fig. 102b) indicated the buoyant density of the latter to be 1.7227 g/cm^3 (64.00% G+C). However, when both strains of R. palustris were compared with M. lysodeikticus (Fig. 102c) the bands due to R. palustris were not separated and gave a combined buoyant density of 1.7236 g/cm^3 (64.89% G+C). The two strains of R. palustris apparently have DNA of almost identical buoyant density suggesting (although not proving) that they are very closely related.

Growth and some properties of phage Rp 1

A vial of freeze-dried phage Rp 1 was resuspended in ATCC medium No. 550 and drops of the suspension applied to a spread plate of R. palustris strain 1e5 and also to a plate of the Rhodospirillum sp. isolated with R. palustris 1e5. In addition, a drop of sterile medium was placed on each spread plate. After photosynthetic incubation of the plates in an anaerobic bag at 30°, 3 to 4 plaques appeared per drop of suspension in the lawn growth of R. palustris. No such plaques were observed on Rhodospirillum or where medium was applied. This indicated that the ATCC stock material resuspended in 0.5 ml contained only about 30 to 40 p.f.u.. A single plaque was removed and used to infect an early exponential culture (10 ml) of R. palustris 1e5, the infected culture being incubated photosynthetically at 30°. Within 2 to 3 d the culture was beginning to lose its turbidity and assumed a "ropy" appearance. A plaque assay of the supernatant medium from such a culture was attempted and revealed only about $0.5 - 2.0 \times 10^4$ p.f.u./ml.

Plaque assays of Rp 1 phage gave rise to a variety of sizes of plaques on any one plate (Fig. 103), but all plaques were clearly visible

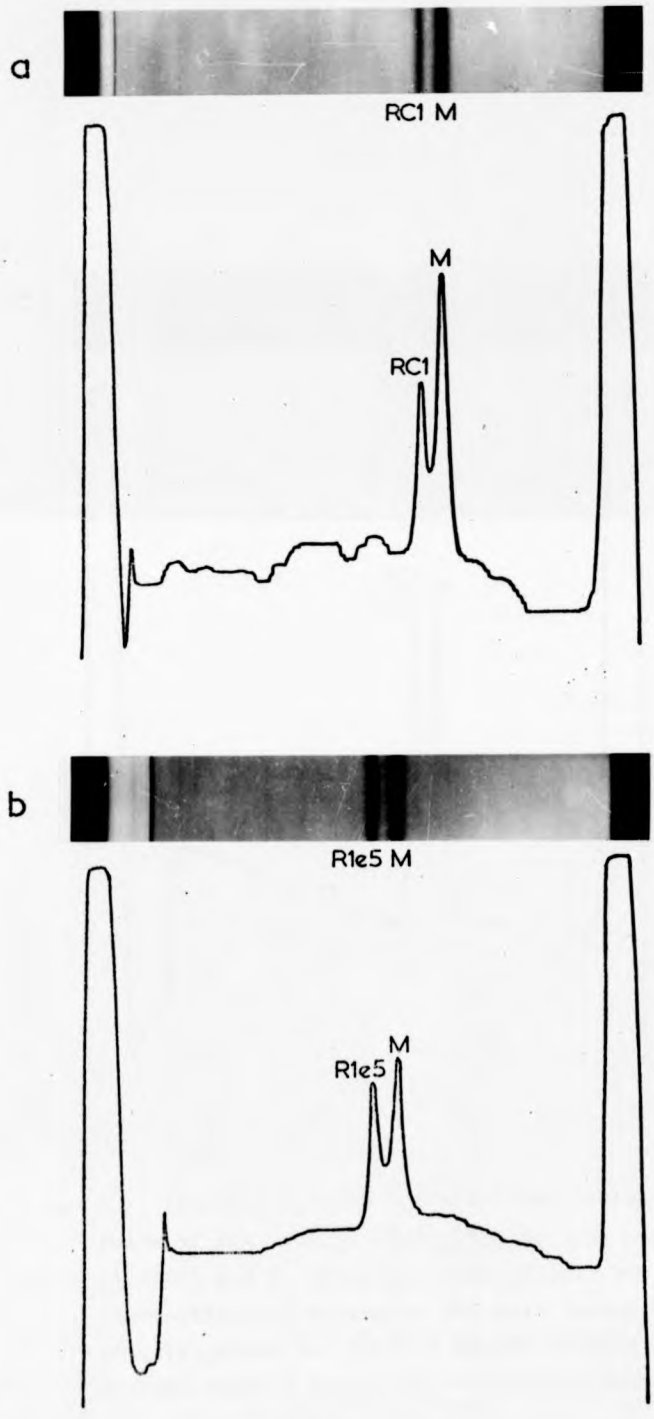
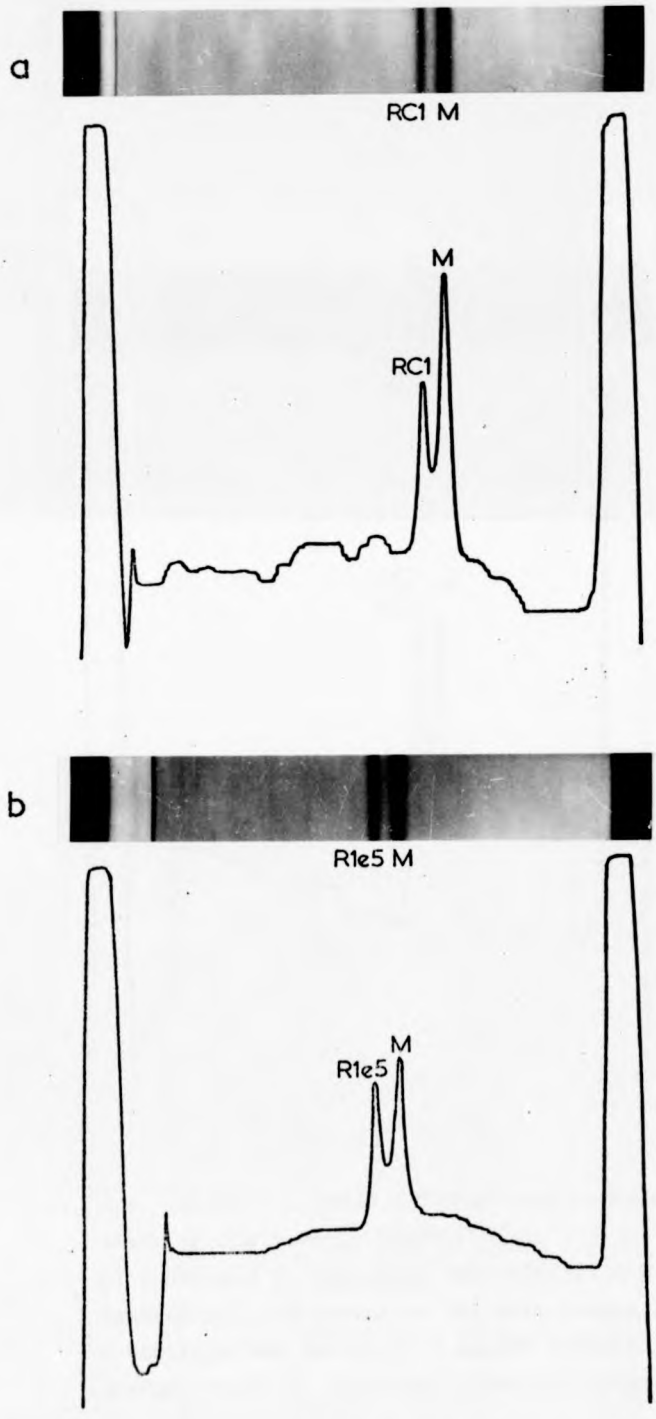


Fig. 102a & b. See page 205 for title and legend.



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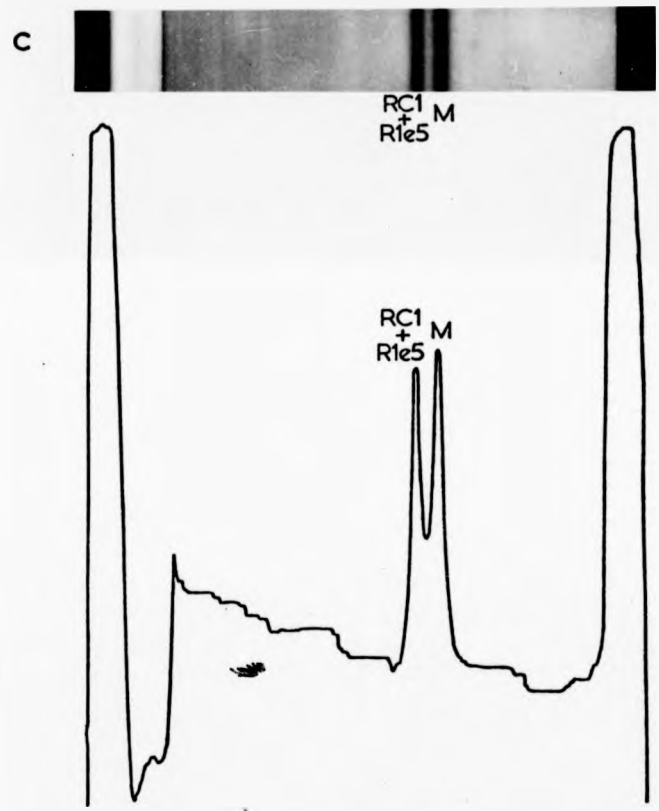

Fig. 102a,b & c. u.v. absorption bands and resulting microdensitometer scans of DNA from M. lysodeikticus (M), R. palustris C1 (RC1) and R. palustris 1e5 (R1e5). The various combinations of extracted DNA were banded by centrifugation for 20 h at 44,000 rev/min on a Beckman Model E analytical ultracentrifuge.

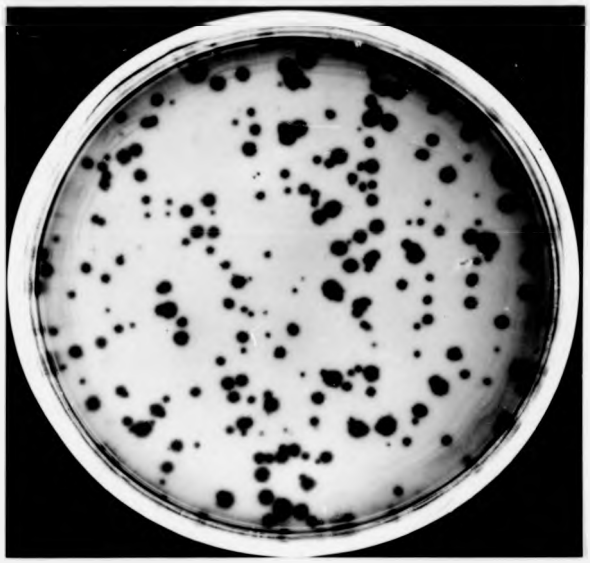

Fig. 103. Plaques of Rp 1 phage in an overlay containing *R. palustris* 1e5 cells. The plate was incubated photosynthetically for 24 h at 30°. Full size image produced by transmission of light through plate onto photographic paper.

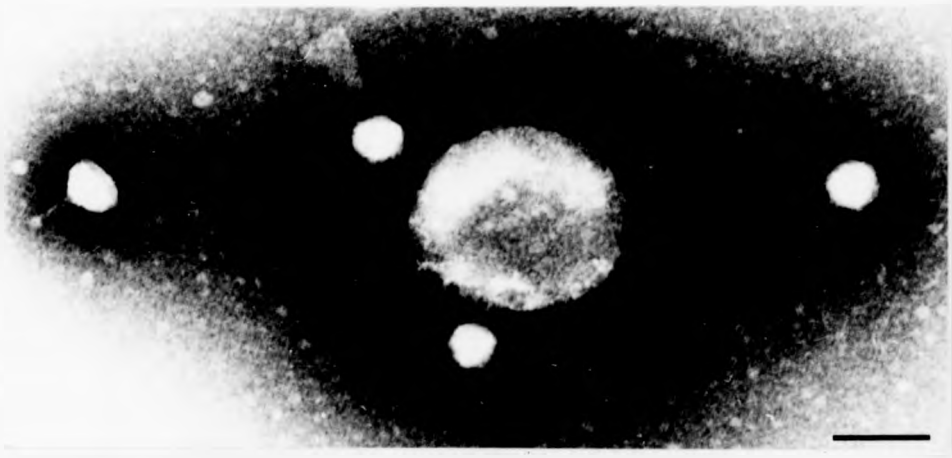

Fig. 104. Negative-stained preparation of Rp 1 phage. Negative-staining with 1% potassium phosphotungstate revealed both empty-headed (dark) and full-headed (light) phage particles mostly absorbed onto vesicle material. Many of the free phage had short tails. Bar represents 0.1 μm.

because of the pigmented nature of the cell growth around them. It was also noted that, occasionally, uppermost plates in the stacks in anaerobic bags gave lower titres than other plates. This appeared to be correlated with either high light intensity or localised heating from the illumination. Consequently, subsequent plaque assays were carried out using illumination by a 60 watt bulb at 30 cm distance (about 800 to 1000 lux) and an adjacent fan operating to maintain the top of the bag at 30°, that is, the temperature of the warm room.

Cultures of R. palustris 1e5 were infected with a progressively higher m.o.i., as more phage containing material became available, until an m.o.i. of 0.1 could routinely be used. Cell concentrations for such infected cultures were initially about 0.5 - 5.0 x 10^8 cells/ml. Lysates from infected liquid cultures could usually be obtained containing up to 1 x 10^8 p.f.u./ml and on rare occasions up to 5 x 10^8 p.f.u./ml, but never any higher.

Examination of centrifuged but not filtered lysates containing high titres of phage, using the electron microscope, revealed small numbers of phage particles most frequently absorbed onto vesicle material (Fig. 104). Complete phage particles consisted of a 65 nm diameter icosahedral head with an 8 to 9 nm diameter, 33 nm long tail. Non-absorbed phage particles were often seen lacking their tails, only having a short stump remaining (Fig. 104). Partially lysed cells revealed the lamellar structure of photosynthetic membranes within them since the potassium phosphotungstate was able to penetrate into the cells (Fig. 105).

The phage Rp 1 was tested for its infectivity of other photosynthetic budding bacteria. Spread plates of R. palustris 1e5, R. palustris C1, R. acidophila and Rm. vannielii were infected with drops of Rp 1 phage suspension and incubated photosynthetically at 30° for 5 d. No lysis of any of the lawn growths, except R. palustris 1e5, was observed.

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Fig. 105. Negative-stained electronmicrograph of R. palustris 1e5 cell lysed by Rp 1 phage. pm, photosynthetic membrane lamellae; f, flagellum; v, vesicular material; p, phage particles. Bar represents 0.2 µm.

Since certain divalent cations such as Ca^{++} have been implicated in absorption of phages of other photosynthetic bacteria, ability of Rp 1 phage to form plaques and to multiply in liquid cultures in the presence and absence of Ca^{++} and Mg^{++} was tested. Plaque assays carried out using overlay agar containing no supplement, 5 mM $CaCl_2.2H_2O$ or 5 mM $MgCl_2.7H_2O$ and using the same phage suspension for each gave rise to titres of 1.2×10^7 p.f.u./ml, $1.0\text{-}1.5 \times 10^7$ p.f.u./ml and $1.5\text{-}2.0 \times 10^7$ p.f.u./ml, respectively. Calcium had no effect on plaquing of Rp 1 phage although magnesium possibly gave rise to slight stimulation. Liquid cultures of R. palustris 1e5 were set up containing no supplements, 5 mM $CaCl_2.2H_2O$ or 5 mM $MgCl_2.7H_2O$ in the PAYE medium. The cultures, containing about 3×10^7 cells/ml, were infected with Rp 1 phage at an m.o.i. of about 0.02. After photosynthetic incubation for 24 h at 30° lysis was observed in the control culture and Mg^{++}-treated culture but was not apparent in the Ca^{++}-treated culture. The remaining cells and debris were centrifuged out of the medium and the supernatant material filtered. The filtered lysate was then plaque assayed. The control culture contained $2.6\text{-}3.0 \times 10^7$ p.f.u./ml, the Ca^{++}-treated culture $5.3\text{-}8.0 \times 10^6$ p.f.u./ml and the Mg^{++}-treated culture $1.6\text{-}2.3 \times 10^7$ p.f.u./ml. Under these circumstances calcium appeared to be repressing lysis of R. palustris 1e5 by Rp 1 phage.

Although lysis of cultures was not consistently observed, on one occasion the lysis of a cuvette culture of R. palustris 1e5 by Rp 1 phage was achieved and followed by optical density changes. A cell suspension containing about 4.3×10^8 cells/ml was incubated in a stoppered, gassed cuvette and growth followed for 2 h by changes in extinction at 670 nm (released photopigment would not interfere at this wavelength). The culture was then infected with phage suspension at an m.o.i. of 0.1. The optical density continued increasing slowly for a further 1.5 to 2 h (Fig. 106) and then dropped for the next hour. There was a shoulder in the drop of

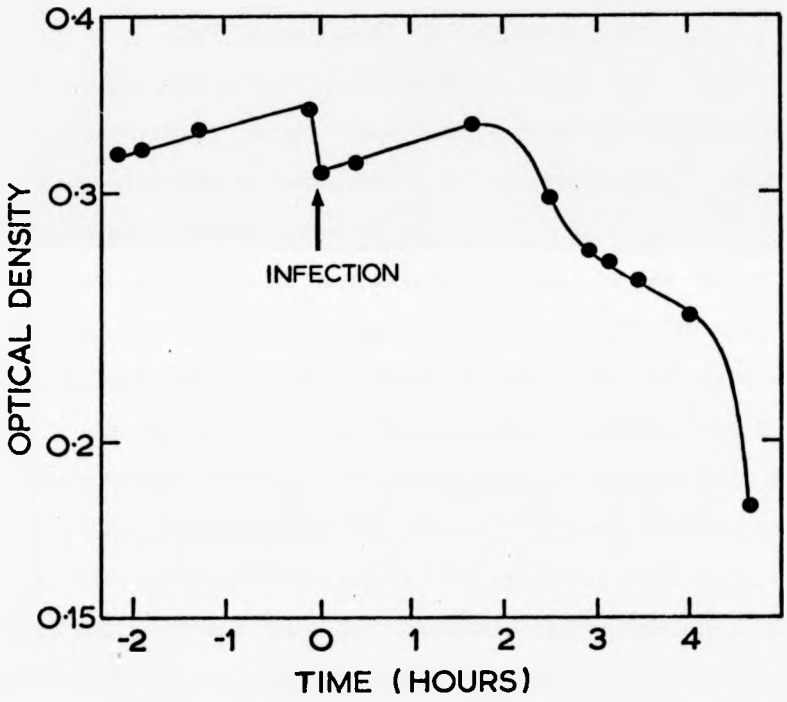

Fig. 106. Optical density of an R. palustris 1e5 cuvette culture before and after infection with Rp 1 phage. Growth of the culture was followed by changes in E_{670} for about 2 h and the culture then infected with phage to an m.o.i. of about 0.1. Optical density changes were then followed for a further 4.7 h.

optical density between 3 and 4 h post-infection. This indicated that the lytic cycle of Rp 1 phage was taking about 2 to 2.5 h and that two such cycles of infection had been followed. It was perhaps significant that on another occasion that the experiment was attempted the growth rate of the culture was considerably higher but no lysis was observed.

Centrifugation of Rp 1 phage in CsCl

Since sufficiently high titres of phage could not be obtained from liquid cultures to permit electron microscopy of absorbed phage on cells, it was attempted to concentrate phage suspensions by centrifuging in CsCl until equilibrium was obtained.

The agar from 45 preparative soft agar plates was combined, liquidised and the agar removed from the slurry by centrifugation (see Materials and Methods). A plaque assay at this stage indicated about 9.1×10^7 p.f.u./ml in the 250 ml of supernate. Material from the supernate was concentrated and banded by centrifugation to equilibrium in CsCl as described in Materials and Methods. Three bands were observed two-thirds of the way down the centrifuge tubes. The uppermost band was pink, the next white and the lowest band the characteristic opalescent bluish-white of a concentrated phage band. All bands were retrieved and pooled (about 60 ml, 1.3×10^7 p.f.u./ml) and dialysed against phage buffer (after dialysis, 1.8×10^8 p.f.u./ml). The dialysed material was further concentrated by CsCl centrifugation and again gave three bands which were this time removed individually. The lowest, opalescent band (about 4 ml) when plaque assayed revealed that it contained less than 10^7 p.f.u./ml. All three bands were dialysed against phage buffer and after this procedure the opalescent band then contained about 1×10^8 p.f.u./ml. All three bands were examined by electron microscopy after negative-staining with potassium phosphotungstate. The uppermost pigmented band (Fig. 107a) revealed numerous vesicles presumably consisting of photosynthetic membrane

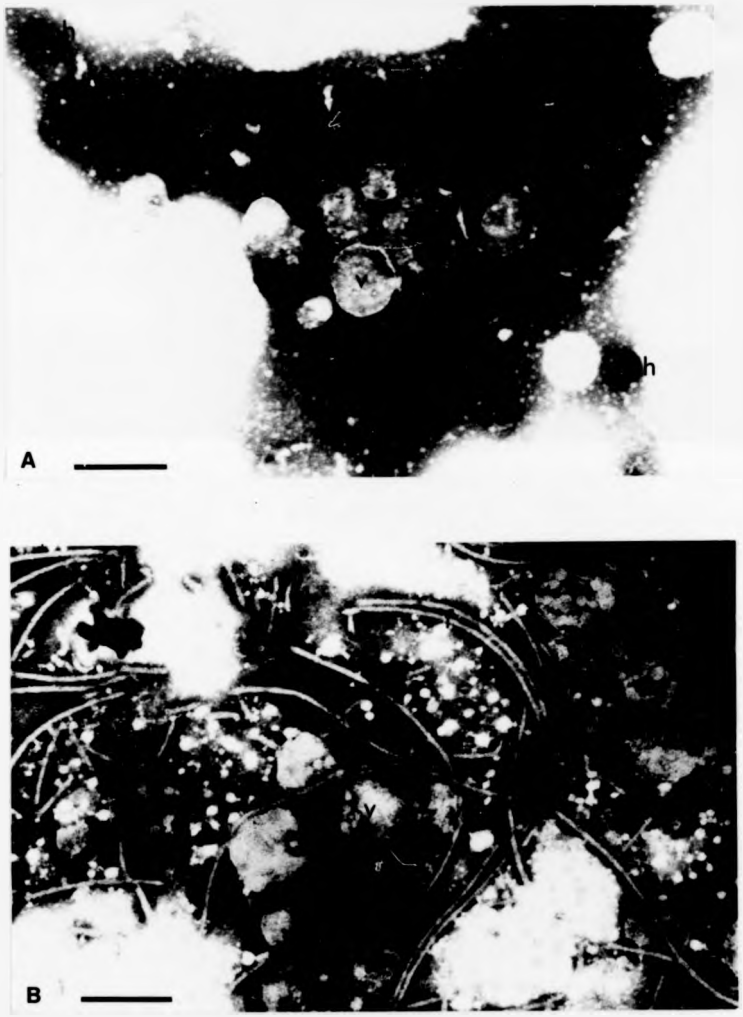

Fig. 107a & b. Electronmicrographs of material banded during CsCl equilibrium gradient centrifugation of lysate. Lysate from Rp 1 infected R. palustris 1e5 was concentrated to form three bands: an uppermost pink band (a), a middle white band (b) and a lower opalescent band revealing no structures. v, vesicular material; f, flagellar fragmen h, empty phage heads. Bars represent 0.2 um.

material. Amongst this material were also a number of empty phage heads. The second white band (Fig. 107b) revealed fragments of flagella and also vesicles presumably of non-photosynthetic membrane material. The phage band in fact revealed no recognisable material. The 1 x 10^8 p.f.u./ml was probably insufficient to be easily found on the electron microscope grid whilst the opalescent appearance of the band was probably due to macromolecular debris which did not show up on negative-staining.

Selection of temperature-sensitive phage

Since Rp 1 phage is apparently not a temperate phage, in order to be able to achieve genetic transfer by the phage it would be necessary to use a conditional mutant unable to carry out intracellular development under non-permissive conditions. In this way infection would be possible without causing lysis of the bacteria and successful transduction might occur. Consequently a t.s. mutant of Rp 1 phage was sought.

Mutagenesis of Rp 1 phage was attempted using nitrous acid. A filtered suspension of Rp 1 phage (about 2-5 x 10^7 p.f.u./ml) was treated at room temperature with sodium nitrite and acetate buffer (see Materials and Methods). Samples were taken at 20 min intervals and immediately diluted for plaque assaying. Even after 100 min treatment of the phage with nitrous acid there was an 87% survival of p.f.u.. Nitrous acid, therefore, did not seem to be a suitable mutagen.

Exposure of Rp 1 phage to u.v. light was found to give a rapid inactivation of phage particles. 0.1 ml samples, taken at time intervals from a stirred phage suspension which was exposed to u.v. light, were diluted and plaque assayed. Fig. 108 shows that treatment up to 2 min gave an approximately logarithmic inactivation. Exposure to u.v. for 1 to 1.5 min at 1.2 x 10^4 ergs/sec/cm^2 gave only 0.1 to 1% survival and would therefore be very suitable for obtaining mutants.

Plates containing numerous plaques were prepared from a

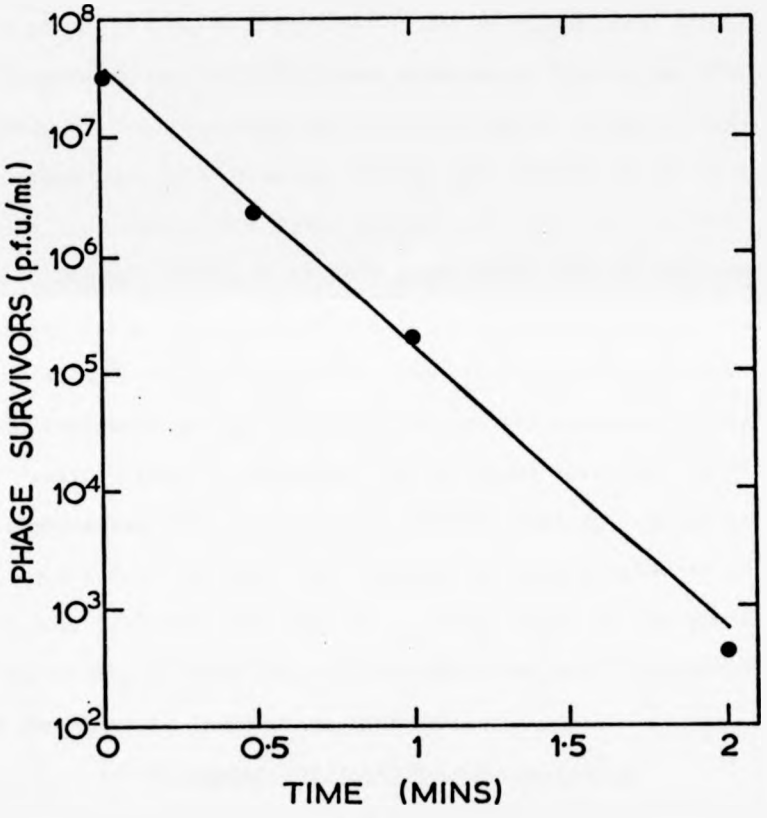

Fig. 108. Survival of infective Rp 1 phage particles during exposure to 1.2×10^4 ergs/sec/cm^2 u.v. light.

suspension of Rp 1 phage which had been u.v. mutagenised for 1 min. Incubation of the plates was at 30° for 2 d. 300 plaques were stabbed with toothpicks and transferred as stabs to duplicate plates overlayed with soft agar and cells, one set of plates again being incubated at 30° and the other at 42°. After 2 d incubation the 30° plates had produced 299/300 plaques whilst the 42° plates produced no plaques at all, even though there had been adequate growth of the cells in the overlay agar. Thus it seemed that Rp 1 phage was either unstable at 42° or it already possessed a t.s. step in the lytic cycle.

Thermal stability of Rp 1 phage particles was examined by comparing the titre of a phage suspension before and after 3 h and 6 h incubation at 30°, 34°, 38° and 42°. The resulting titres after these treatments indicated negligible effect of the temperatures on the titres up to 38° whilst after 6 h treatment at 42° there were still 67% of infective particles surviving. This indicated that Rp 1 phage was not very thermolabile at 42° and that inability to form plaques at 42° must be due to some inherent t.s. step in the lytic cycle of the phage. It remains to be seen at what stage in the infection and multiplication the phage is sensitive to temperature increases.

Attempted conjugation in R. palustris

In order to test whether any genetic transfer by conjugation could be achieved, exchange of antibiotic markers in cultures of two mixed strains of R. palustris was examined.

A culture of R. palustris C1 rif^R 2 (see p. 196) was grown up overnight in PAYE medium containing 100 µg/ml rifampicin. A NAL-resistant mutant of R. palustris 1e5 was selected from survivors of a NAL-treated culture and grown in 50 µg/ml NAL overnight. 0.1 ml of R. palustris C1 rif^R 2 and 0.1 ml of R. palustris 1e5 nal^R were spread as a mixture on a PAYE agar plate. A second plate was spread with 0.15 ml of the rif^R strain

and 0.05 ml of the nal^R strain. Two other plates were spread with 0.1 ml of each of the strains individually. The plates were then incubated photosynthetically at 30° for 3 d. After incubation the cells from each plate were washed off and resuspended in 2 to 3 ml PAYE. Serial dilutions of each cell suspension were plated on PAYE agar to give a total cell count and on PAYE agar plus 50 µg/ml NAL and 100 µg/ml rifampicin to give numbers of doubly resistant isolates. All cell count plates were incubated photosynthetically at 30° for 6 d. Table 10 shows an analysis of the cell counts obtained. Using the figures of spontaneous mutation frequency obtained for the pure cultures of the two strains, spontaneous mutation frequencies for 1:1 and 3:1 proportions of mixed strains were calculated and compared with the experimental results obtained. The frequencies for doubly resistant isolates were in fact very close to those expected if only spontaneous mutation had occurred. Thus there was no evidence of genetic transfer having occurred.

DISCUSSION

Although little information was gained about the differentiation of R. palustris, mutagenesis and genetic transfer studies did demonstrate that the organism has the potential of offering morphological mutants (including t.s. mutants) as well as a possible means of genetic transfer using the naturally t.s. phage Rp 1. However, a number of results obtained are worthy of discussion in their own right.

It was interesting to note that two mutants were isolated which apparently had weakened cell walls in the region of the mother and daughter cell bodies but not the tube, and that one such mutant was in fact t.s.. These mutants indicated that there was a difference in the

Table 10. Analysis of attempted genetic exchange between R. palustris C1 and R. palustris 1e5. Strains were spread on PAYE agar plates and grown photosynthetically for 3 d. The resulting cultures were examined for total viable counts and counts of doubly resistant mutants.

R. palustris strains	total count (cells/ml) PAYE	selective count (cells/ml) PAYE + NAL + rifampicin	recombination or spontaneous mutation frequency
C1 rif^R	2.68×10^{10}	8.2×10^4	3.05×10^{-6} (a)
1e5 nal^R	4.45×10^9	9.9×10^2	2.22×10^{-7} (b)
C1 rif^R + 1e5 nal^R (1:1)	3.45×10^{10}	4.19×10^4	1.22×10^{-6} ($*1.64 \times 10^{-6}$)
C1 rif^R + 1e5 nal^R (3:1)	2.32×10^{10}	5.75×10^4	2.47×10^{-6} ($+2.34 \times 10^{-6}$)

*, calculated frequency assuming no recombination, $\frac{a + b}{2}$

+, calculated frequency assuming no recombination, $\frac{3a + b}{4}$

wall structure between cell bodies and the tube. It can be envisaged that some component of the cell body wall could not be synthesised giving rise to a weakened cell wall, whereas the tube does not contain this component, or it is not essential for the rigidity of the tube wall. The alteration that occurred in the cell wall need only have been a slight one. Indeed, rod to coccal morphogenesis in A. crystallopoietes is thought to be due to insertion of two glycine molecules in glycopeptide cross-links and the glycopeptide backbones being shortened to one-third of their original chain length (Krulwich et al, 1967a,b).

The additional pigment mutation observed in the t.s. cell wall mutant would seem to be similar in nature to the green mutant of R. palustris, possessing precursors of bacteriochlorophyll, used in the studies of Solov'eva & Fedenko (1970). They were able to demonstrate that although their mutant produced pigment in the light it contained no photosynthetic membrane lamellae. In the same fashion as the yellow mutant described here their green mutant was unable to grow under anaerobic conditions indicating the loss of ability to photosynthesise. Clearly this mutant would not have been isolated if the original mutant isolation had been carried out photosynthetically. In addition, pigment synthesis must be induced by illumination and not low oxygen tensions. Unfortunately, it is not possible to say whether the yellow pigment induced by illumination was a precursor of carotenoids or of bacteriochlorophyll. The presence or absence of carotenoids certainly does not confer presence or absence of photosynthesis. Sistrom (1966) investigated photochemical properties of mutants of R. spheroides some of which carried out photosynthesis with carotenoids and some without carotenoids. On the other hand some mutants could not photosynthesise regardless of whether or not they possessed carotenoids. It seems likely, however, that a mutant containing no bacteriochlorophyll or only chlorophyll precursors might not be able to

photosynthesise. It is interesting to note that, in complete contrast, Wittenburg & Sistrom (1971) were able to isolate a mutant of R. spheroides which grew normally under photosynthetic conditions but which was unable to grow exponentially under aerobic conditions.

It was disappointing to find that rif^R mutants of R. palustris did not exhibit any morphological abnormalities. Perhaps mutants giving rise to both resistance to rifampicin as well as modified development might most commonly be lethal. Although some rif^R B. subtilis mutants have altered spore morphology many are also completely blocked in sporulation (Doi et al, 1970; Sonenshein & Losick, 1970). Perhaps in obligatory developmental systems such as R. palustris more information may be gained if conditional rif^R mutants were available. It is possible that all the selected mutants were in fact non-permeable to rifampicin and the RNA polymerases were unaltered. One way in which this might be detected is by following m-RNA or protein synthesis before and after making cells permeable with toluene. Another explanation of the results might be that RNA polymerase changes may not be involved in the developmental control of R. palustris. This would seem to be so in Caulobacter where three developmental stages have been found to contain identical RNA polymerases (Bendis & Shapiro, 1973).

Andrews (1973) was able to overcome problems of apparent insensitivity of S. faecalis to trimethoprim in presence of thymine when selecting for thy^- mutants by pre-incubating a culture in minimal medium containing trimethoprim but no thymine. This apparently then gave thy^- mutants a selective advantage over wild type cells. Such a procedure was unsuccessful with R. palustris, the growth of the organism being highly insensitive to the folate antagonist. Wickner (1974) found that d-TMP-requiring mutants of Saccharomyces cerevisiae could be selected by growth in the presence of sulphanilamide (folic acid analogue), aminopterin (often used as an alternative to trimethoprim) and d-TMP. Apparently

trimethoprim would not substitute for aminopterin in this system and this would indicate that they have slightly different modes of action. It would be interesting to attempt to select d-TMP-requiring mutants of R. palustris by this method as well as testing whether aminopterin might permit thy⁻ selection by the more conventional methods.

Studies on R. palustris involving phages had to be carried out using Rp 1 phage, since attempts at isolating new phages were unsuccessful. In turn this dictated that R. palustris 1e5 had to be used as host because of the strain specificity of Rp 1 phage (Freund-Mölbert et al, 1968). Apart from the fact that it had a shorter tube it was particularly evident that R. palustris 1e5 had a larger diameter, kidney-shaped mother and swarmer cell body, although the tube was similar in diameter to that in R. palustris C1. If it is true to say that chemical change in the wall composition might be reflected in cell shape it is perhaps significant that Framberg et al (1974) demonstrated three serotypes of R. palustris which correlated with three types of lipopolysaccharide found in the strains (Weckesser & Drews, 1973). Not only might other strain differences in the wall composition have a bearing on the shape of R. palustris strains but it is also easy to see that R. palustris 1e5 might have a group of unique sugars in its lipopolysaccharide acting as the phage receptor site.

The major problem faced in the experiments with Rp 1 phage was that of inconsistently observed lysis of liquid cultures of R. palustris 1e5 giving relatively low titres of phage. Bosecker, Drews & Jank-Ladwig (1972) claimed to be able to get titres of 5 - 7 x 10^9 p.f.u./ml after infecting 3 - 4 x 10^8 bacteria/ml with phage at an m.o.i. of 0.05 to 0.1, and incubating for 14 to 16 h at 30°. Using these very same conditions it was found impossible to obtain such high phage titres. It is possible that PAYE medium had an inhibitory effect on Rp 1 infection, growth or lysis although addition of supplementary Ca^{++} or Mg^{++} did not increase titres as

they have been shown to for a phage of R. capsulata (Schmidt et al, 1974). Rp 1 phage has also been shown to absorb to intracytoplasmic membranes of host cells (Freund-Wolbert et al, 1968; Bosecker, Drews & Jank-Ladwig, 1972; Bosecker, Drews & Tauschel, 1972). Similar observations were made in this study and it is possible that the ionic composition of the medium might have caused absorption onto membranes to a greater extent than was observed by the other workers.

Attempts at concentrating Rp 1 phage by centrifugation in CsCl were most unsuccessful. The banded, harvested infective phage particles obtained after the final dialysis accounted for only 2% of the infective particles initially loaded into the centrifuge tubes. Removal of CsCl from harvested bands by dialysis in fact increased the titre 10-fold. Thus it appeared that CsCl was causing a partially reversible denaturation of the infective phage particles. Although it was clearly demonstrated in this study that a considerable titre of phage was lost during centrifugation in CsCl, Bosecker, Drews & Jank-Ladwig (1972) used a discontinuous CsCl gradient to demonstrate that Rp 1 phage contained DNA. Although they indicated diagrammatically that phage was banded at the bottom of the centrifuge tube there was no real evidence of this as titres which they presented only showed a gradient of phage titre rather than a band. In their studies of the R. capsulata phage, Schmidt et al (1974) have described methodology used for phage concentration either from confluent lysis plates or liquid culture lysates but without using gradients during concentration by centrifugation. Perhaps these methods might be useful for Rp 1 phage. If higher titres of Rp 1 phage can be obtained it will then be possible to investigate the localised absorption of the phage, first demonstrated by Bosecker, Drews & Tauschel (1972), in a synchronous culture system, in addition to using the phage as a transducing agent.

FUTURE PROSPECTS

Under standard growth conditions for R. palustris quite consistent and defined morphology could be observed, in spite of the fact that altered environmental conditions could result in changes of features of cultures. Using such defined conditions R. palustris could easily be studied growing in synchrony, especially during the first generation. Not only did the organism reveal a distinctive cell cycle with periodic morphological and ultrastructural changes which could be considered as differentiation at the lowest level, but also cell division gave rise to two distinct cell types; unmistakably a process of cellular differentiation.

Although most of the morphological and ultrastructural changes in the cell cycle were examined and patterns of gross protein, DNA and possibly RNA synthesis were followed, it is true to say that a considerable amount has yet to be learnt about the cell cycle of R. palustris.

Since photosynthetic membranes are synthesised de novo in daughter cells studies on membranes and photopigments should reveal much information. For instance, it may be possible to discover whether the de novo synthesis is triggered by the cell attaining a certain mass or by accumulation or dilution of activators or repressors. It may also be possible to establish whether development can continue in the absence of membrane and pigment synthesis under photosynthetic conditions thus giving rise to a photosynthetic mother cell and a non-viable obligately aerobic daughter. When aerobically dark grown R. palustris cells are transferred to anaerobic illuminated conditions the organism adapts to the new conditions by synthesising photosynthetic membranes (Tauschel & Drews, 1967) and the appropriate photosynthetic metabolic apparatus. One intriging possibility that arises with the availability of synchronous cultures of R. palustris is that of inducing a change to photometabolism in a synchronised aerobic culture. Ultrastructural and physiological changes could be followed during the cell cycle to determine at what stages during the cycle the various

synthetic processes may occur. Presumably the syntheses giving rise to de novo photosynthetic apparatus in the daughter cell during normal photosynthetic growth would be the same as those involved in the induction of pigments, membranes and new metabolic pathways in a cell previously grown aerobically. It would be interesting to know, therefore, if in the latter case the photosynthetic apparatus is first synthesised in the daughter cells only. Such studies would require methods of following the synthetic processes. The incorporation of radioactive-labelled membrane phospholipid precursors might be possible during synchronous culture. Incorporation of δ-aminolevulinic acid as a precursor of bacteriochlorophyll has not so far been achieved in R. palustris, though modification of experimental conditions might make this possible. The use of radioactive mevalonic acid to indicate carotenoid synthesis has been possible in some bacteria (Britton & Goodwin, 1971) but remains to be attempted in R. palustris.

The synthesis of various enzymes during the cell cycle has also been considered by a number of workers (Halvorson et al, 1971). Most relevant to this examination of R. palustris as a model of differentiation are the studies by Ferretti & Gray (1967,1968) of enzyme synthesis in synchronous cultures of R. spheroides. Although one would anticipate quite different patterns of enzyme synthesis in R. palustris as a result of the de novo synthesis of photosynthetic apparatus in daughter cells, it is probable that the assays they used for δ-aminolevulinic acid synthetase, δ-aminolevulinic acid dehydrase, succinyl CoA thiokinase, alkaline phosphatase and ornithine transcarbamylase might all be applicable. Such experiments would depend on a high enough concentration of synchronous cells being available. It is possible that it would be necessary to pool swarmer cells from a number of sucrose gradients before synchronous growth or to pool cells from the same developmental stage from a number of synchronously

growing cultures. Alternatively, the development of a zonal rotor technique (Lloyd et al, 1975) for R. palustris might be necessary to obtain sufficient cells to allow enzyme assays to be carried out during synchronous growth from the swarmer phase.

It would seem from experiments in this thesis that observations on cell morphology and optical density were insufficiently sensitive or specific to allow examination of possible stable m-RNA translation after inhibition of transcription. Using the patterns obtained for enzyme synthesis, if periodic, as well as such defined features as bacteriochlorophyll carotenoid and membrane production it should be possible to carry out experiments with rifampicin and chloramphenicol examining R. palustris for synthesis of long-lived m-RNA and control of the cell cycle at the translational as well as transcriptional level.

The patterns of synthesis of enzymes and other proteins could also be followed after separation by gel electrophoresis of protein fractions obtained at different stages of the development in the cell cycle (Scott, 1971). Here, almost certainly it would be necessary to obtain large scale synchronous cultures using a zonal rotor. Provided it was possible to determine which bands refered to which proteins such techniques would allow direct comparison of the appearance and disappearance of certain cellular components.

Experiments with NAL yielded much information on the discontinuous pattern of DNA synthesis as well as its association with cell division and elongation. Although results obtained for incorporation of ^{32}P into alkali-resistant material (DNA) were largely in accordance with results from NAL-treated cultures a more specific assay procedure for DNA would be desirable. Even if thy$^-$ mutants could not be obtained a very wide range of DNA precursors still remain to be tested under a variety of conditions.

The only antibiotics which gave significant results by

inhibition of specific phenomena in the cell cycle of R. palustris were penicillin and NAL. However, if further inhibitors could be found which, for instance, inhibit photopigment synthesis or membrane synthesis it might be possible to determine interdependence of more cell cycle events. Similarly, t.s. mutants would be invaluable and it is clear from the results obtained here that such mutants can be obtained.

One of the ultimate aims in studying any model system of differentiation would be to gain an understanding of the complete genetic control of the organism. Even in organisms as well studied as E. coli knowledge of genetic control is by no means complete, but some progess has been made in genetical studies of spore formation in Bacillus (Szulmajster, 1973). Along with mutants of R. palustris, genetic transfer systems would be desirable. So far, transduction using a t.s. phage looks the most promising approach although it is possible that under suitable conditions transformation may be possible.

In addition to their uses in genetic transfer, isolation of phages specific for R. palustris might also prove invaluable in providing precise markers of synthetic events both with respect to time and location. Thus measurement of absorption of phage which are specific for flagella would give a means of determining the timing of flagellar synthesis through the cell cycle. On the other hand, the location of absorption of phage to available cell wall receptor sites as observed by electron microscopy could demonstrate the precise location of these sites on the cell wall through the different stages of the cell cycle. However, this would depend on being able to make available high titres of phage because of the high m.o.i. required to visualise absorbed particles.

Because of the symmetry of growth and division that bacteria normally display the phenomenon of aging of organisms which display polar or uni-directional growth has largely been overlooked. It has already

been pointed out that R. palustris exhibits ageing. As soon as a swarmer has lost its flagellum, synthesised its holdfast and then its tube it is apparently capable of sequentially producing numerous swarmers on the end of the tube but without losing its own identity. It is difficult to devise an experiment whereby one could determine just how many swarmers a mother cell is capable of producing. It is certainly not possible to follow swarmer release from a mother cell using a slide culture as microcolonies soon become congested. One possible approach could be to use a technique similar to that of the synchronisation procedure of Helmstetter & Cummings (1963, 1964). If first generation mother cells could be absorbed onto a permeable substrate and swarmers eluted off with nutrient medium it is reasonable to expect that released swarmers could be detected throughout the "life" of the mother cells. Preliminary investigations were in fact pursued using such a method but a suitable membrane material which allows specific attachment of holdfasts has yet to be found.

Not only must R. palustris be further considered as a differentiating organism alone but also in the light of observations of differentiation and its control in other bacteria. Within the gradient of morphogenesis displayed by the budding Rhodospirillaceae the simplest member, R. acidophila, is probably now the least well understood. Once a method of synchronisation of this organism has been found a similar approach of defining morphological, ultrastructural and physiological events in the synchronous cell cycle must be pursued and then followed by studies of their control.

This study on R. palustris has shown the organism to be intriguing in itself and has also extended our knowledge of differentiation in the group of budding photosynthetic bacteria. It is not difficult to see how the range of complexity displayed by them could be extrapolated to the relationships between wide ranges of organisms from bacteria through to blue-green algae and yeasts through to filamentous fungi.

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