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Sporulation competence in
Physarum polycephalum CL

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Presented for the degree of Doctor
of Philosophy in the Faculty of Science,
University of Glasgow

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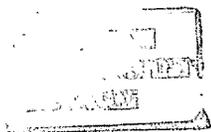
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AILEEN J. CHAPMAN

To my family

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SUMMARY

The central aim of this work was to explore the possible value of P. polycephalum CL as a model of eukaryotic differentiation. Initially it was hoped to obtain a method of reproducibly obtaining sporulation and later to analyse sporulation both biochemically and genetically.

Microplasmodia of the CL strain were found to yield the highest sporulation frequencies when harvested at the end of exponential growth. Sporulation frequencies of microplasmodia harvested at other points in the growth cycle could be improved by allowing overnight growth, as a surface plasmodium, before exposure to starvation medium. The minimum length of starvation in the dark required before one light pulse would induce sporulation of all plasmodia was found to be 72 h.

Plasmodia became committed to sporulation about 4 h after illumination. The time of commitment to sporulation was related to the time of illumination, and not the overall length of starvation, since altering the time of illumination also altered the time of commitment.

The importance of DNA replication and mitosis during the process of sporulation was assessed by examining the effects of inhibitors of these events on starving plasmodia. Nocodazole, an inhibitor of microtubule assembly, prevented sporulation if added any time up to 48 h during starvation. It was assumed, therefore, that the last mitosis during starvation occurred at about 48 h. However, nocodazole might also have affected some other event, besides mitosis, which involved microtubules.

The DNA synthesis inhibitor, hydroxyurea, prevented sporulation if added at any time up to 24 h during starvation. This suggested that the last DNA replication preceded the last event susceptible to inhibition by nocodazole by some 24 h.

By pulse labelling with [methyl-³H] thymidine during starvation, the periods of DNA synthesis during the 72 h starvation period were defined. Periods of DNA replication began at about 4 h, 15 h and 24 h during starvation, confirming that the last replication occurred at about 24 h and demonstrating that this was the third replication to occur during starvation.

The patterns of DNA replication in a sporogenous and an asporogenous culture were compared in an attempt to further clarify the role of DNA replication during sporulation. In the asporogenous derivative, the third period of DNA synthesis, normally detected in the wild type strain, did not occur, yet the previous two rounds of DNA synthesis took place normally. The asporogenous strain was produced by continuous subculture of microplasmodia in broth medium. Before the strain became fully asporogenous, it showed a delay in response to light before it would sporulate. Thus the strain only sporulated after a light pulse at 96 h instead of the normal 72 h. There was, in this strain, a concomitant delay in the escape of the plasmodium from nocodazole inhibition of sporulation. Thus the final replication at about 24 h during starvation and the nocodazole sensitive event which followed some 24 h later were important for determining the condition of the plasmodium for response to light and advance to sporulation.

Microplasmodial cultures were able to grow in the presence of both hydroxyurea and the protein synthesis inhibitor, cycloheximide, at concentrations which normally inhibited growth, if incubated in their presence for extended lengths of time. This may have been due to instability of the drugs at the incubation temperature or P. polycephalum may have been capable of breaking these drugs down. Similar results were obtained with nocodazole, but in addition microplasmodia also developed resistance to the drug.

A method of isolating sporulation deficient mutants was developed and several such mutants were obtained. In a preliminary genetic analysis of sporulation a cross between sporogenous (Spo^+) amoebae and asporogenous (Spo^-) amoebae was made. Although the diploid Spo^+/Spo^- plasmodium sporulated, none of the progeny able to form plasmodia in clones (matAh) were able to do so. Sporulation capacity of Spo^+/Spo^- heterokaryons formed by fusion of plasmodia was also investigated, in these asporogeny was dominant. Although no definite results were obtained from the genetic aspect of this work it has provided a base for further genetical studies on the process of sporulation in P. polycephalum CL.

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Abbreviations

AraC	1- β -arabinofuranosyl cytosine
BuDR	5-bromo-2'-deoxyuridine
DMSO	Dimethylsulphoxide
DM-1	Defined medium-1
DNA	Deoxyribonucleic acid
DSDM	Dilute semi-defined medium
E ₄₀₀	Absorbance of a solution in a cell of 1 cm light path at a wavelength of 400 nm
LIA	Liver infusion agar
LIB	Liver infusion broth
No	Number
NTG	N-methyl N'-nitro N-nitroso guanidine
psi	pounds per square inch
SDM	Semi-defined medium
SM	Sporulation medium
TCA	Trichloroacetic acid
ts	temperature-sensitive

INTRODUCTION

The genus Physarum belongs to the class Myxomyceteae, phylum Myxomycophyta. Organisms in this class lack cell walls during the vegetative stage of their life cycle and exhibit amoeboid motion. This vegetative stage in Physarum is called a plasmodium and is most easily found in nature on rotting trees or leaves or in the soil. Culture of myxomycetes in the laboratory has been possible for more than 100 years; originally they were grown on their natural substrates in moist chambers. Lister (1888) reported growth of myxomycetes on several species of fungi. Fungi proved to be satisfactory substrates for several myxomycetes but the crude methods used in these cultures resulted in many of them being contaminated. Howard (1931a,b; 1932), Camp (1937), Cohen (1939, 1941) and Sobels (quoted in Daniel & Rusch, 1961) reported the isolation of pure cultures which grew on autoclaved yeast or oats and Hok (1954) obtained growth on autolysed yeast preparations. The disadvantage of these methods was that sustained growth was being achieved only on insoluble natural substrates which were unsuitable for nutritional and biochemical investigations. This had led to a general belief that particulate matter was essential to the nutrition of myxomycetes. Only when pure culture methods were developed was it shown that myxomycetes were capable of growing on a soluble substrate. Daniel and Rusch (1961) published a method for growing Physarum polycephalum on semi-defined soluble medium. This contained chick embryo extract as an absolute requirement for growth but later it was replaced by hematin (Daniel et al, 1962).

Before pure culture was possible, interest in the myxomycetes had been sporadic and mostly limited to taxonomic and morphological studies. Reviews by Martin (1960) and Alexopoulos (1960, 1963)

summarize the large number of contributions on taxonomy and morphology made up until that time. Once pure culture of P. polycephalum became an established technique, then biochemical analysis of many aspects of the life cycle also became possible.

1. - The life cycle of P. polycephalum

The life cycle of P. polycephalum was first described by Howard (1931a) and is illustrated in figure 1. It consists of alternating haploid and diploid generations. The main vegetative stage is the diploid plasmodium which is a yellow, flat mass of multinucleated protoplasm (Plate 1). Within a plasmodium the protoplasm can easily be seen streaming rhythmically in alternating directions through a fine network of veins. Although protoplasmic streaming is quite apparent there is no appreciable movement of a plasmodium during growth. During growth all the nuclei in a plasmodium undergo synchronous mitosis (Howard, 1932). A plasmodium will continue to grow as long as nutrients are present but when limited by lack of nutrient, differentiation occurs. Two different pathways of differentiation are open to the starved plasmodium. 1) In the absence of light, starved plasmodia undergo cell cleavage without ~~mitosis~~^{meiosis} thus becoming encysted, forming thick walled dormant structures or sclerotia. Microplasmodia form similar diploid dormant stages called spherules. 2) In the presence of light, starved plasmodia form sporangia (Plate 2). During this process (Plate 3) the diploid nuclei undergo meiosis and haploid spores are formed within sporangia. It is only here at the end of sporangium or sclerotial formation that the organism may be considered as being multicellular; at all other times in the life cycle it is either acellular or unicellular. Spherules or sclerotia will give rise to plasmodia again when sufficient nutrient is available. Spores,

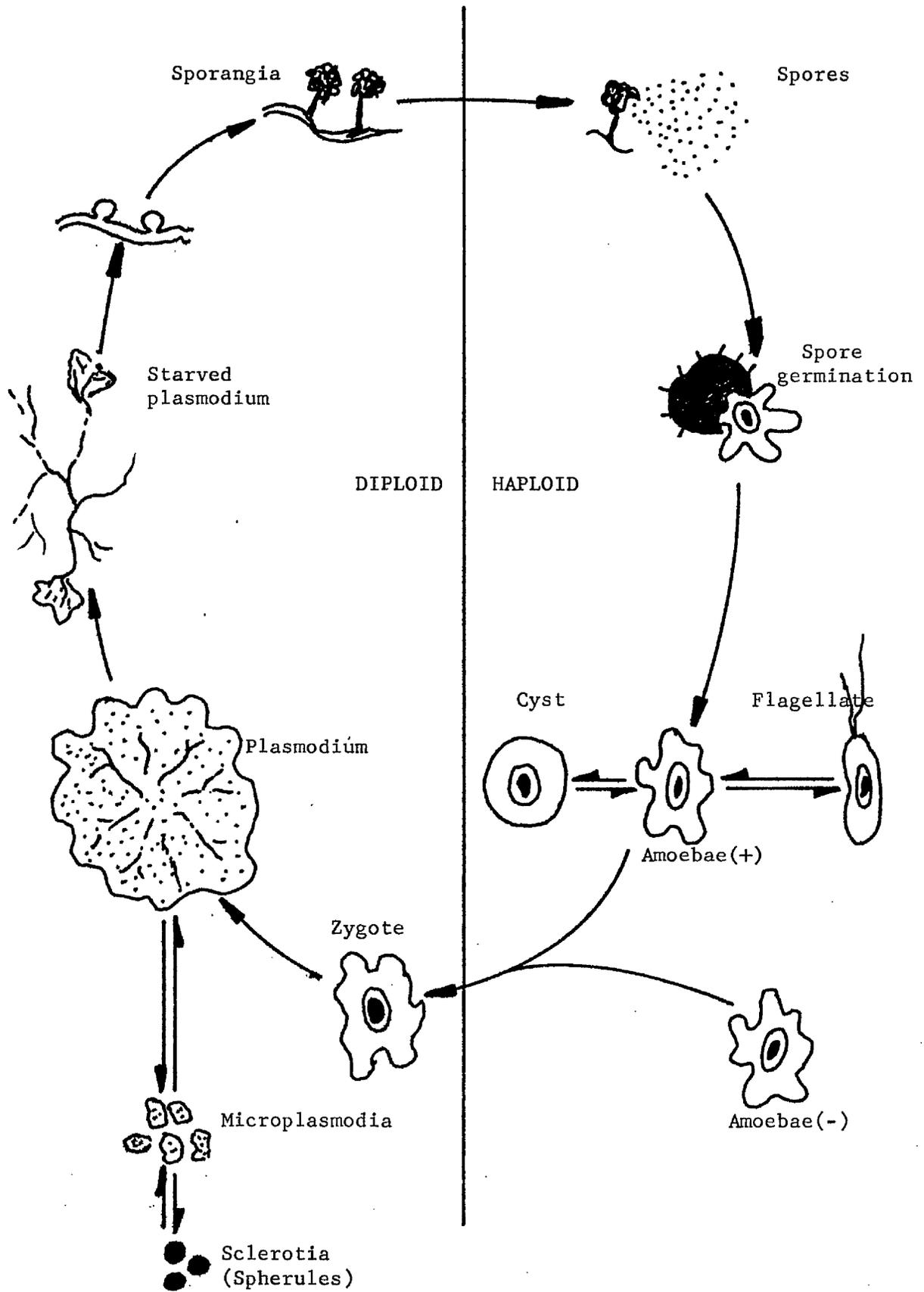


Plate 1

Photograph of a growing plasmodium on SDM agar (Appendix 1)
in a 9 cm petri dish.



Plate 2

Photograph of sporangia formed after illumination of a starved plasmodium on DSDM agar (Appendix 1).



Plate 3

Stages of sporulation of *P. polycephalum*

- a) When plasmodia are starved they change appearance migrating and extending thin yellow veins of protoplasm. Within 12 to 16 h of illumination small nodules become apparent at intervals along the veins. These arise simultaneously.
- b) Within about 1 h of being formed, these nodules extend upwards becoming small pillars; veins become darker in colour.
- c) Tip of pillar becomes swollen as stipe becomes constricted; the tip is a pale yellow colour as most of the pigment has been left behind in the veins which are now a hardened residue called the hypothallus.
- d) Large number of nuclei in the head degenerate (Guttes et al, 1961) whilst those remaining undergo a final meiosis, the mature sporangia become encapsulated and deposition of melanin pigment occurs.

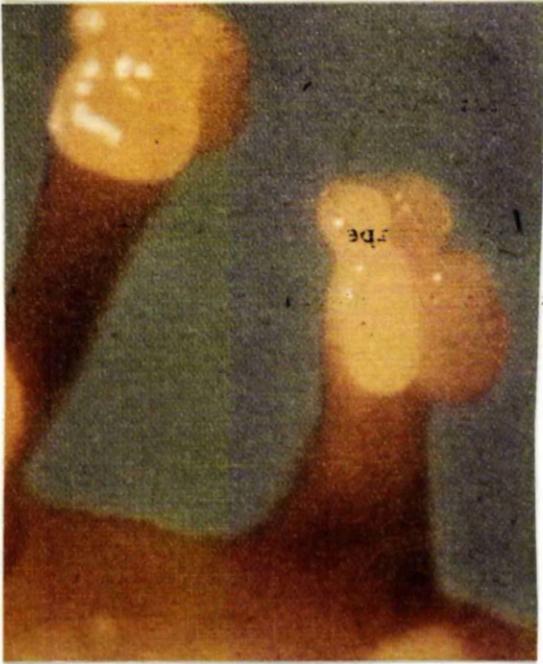
a)



b)



c)



d)



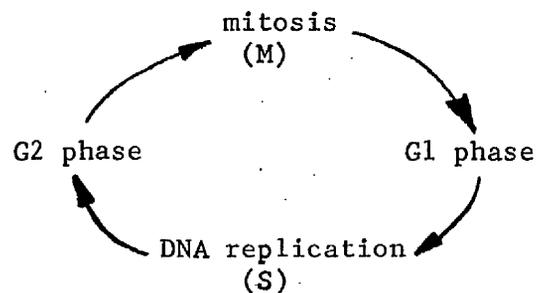
Photographs from Cummins & Rusch, 1968.

however, generally contain single haploid nuclei which form amoebae on germination in moist conditions. The haploid amoebae will transform into motile swarm cells with one or two flagella in more fluid environments and can survive adverse conditions by becoming encysted (Rusch, 1970). Two amoebae (or two flagellated cells) give rise to a plasmodium by fusing to form a diploid zygote. Dee (1962, 1966b) demonstrated that two amoebae can only fuse if they are of different mating types. From zygotes, after nuclear fusion, a diploid plasmodium arises by nuclear division accompanied by growth of protoplasm. There is no accompanying cell division and consequently the plasmodium can be considered as one large multi-nucleated cell. Guttes et al (1961) published a detailed description of the morphology of the life cycle of P. polycephalum in pure culture.

2. The cell cycle of P. polycephalum

a) The eukaryotic cell cycle

The cell cycle of all eukaryotes can be represented as:-



The complete cycle usually consists of four stages labelled G1, S, G2 and M. The first three were originally believed to be a single interphase or period of rest between mitoses. However it was later shown that, although protein and RNA synthesis occurred throughout, DNA synthesis was restricted to one part of interphase called the S phase

which was separated from mitosis by two G phases (growth or gap phases).

Once DNA replication has occurred, cells will normally proceed uninterrupted through the other stages of the cell cycle. Cells not destined for immediate division are generally arrested in G₁.

P. polycephalum differs from the majority of eukaryotes, which have been studied, in that its cell cycle lacks a G₁ phase; DNA synthesis starts immediately after mitosis (Nygaard et al, 1960). However, the absence of G₁ has been reported in other rapidly dividing cells, sea urchins, grasshopper embryos and plant root tips being examples of such cells (Graham & Morgan, 1966). Furthermore it has been demonstrated that under optimal cultural conditions, cells of a Chinese hamster cell line have no G₁ phase in their cell cycle (Rao & Sunkara, 1980). It is suggested that by retaining a high level of protein synthesis during mitosis, the factors necessary for the initiation of DNA synthesis are manufactured whilst the cells are still in mitosis (Rao & Sunkara, 1980). However, under subnormal conditions, protein synthesis during mitosis becomes inefficient and a G₁ period takes place to allow synthesis of inducers of DNA replication.

b) Mitosis in P. polycephalum

i) Mitotic synchrony

The stages of mitosis and their duration in P. polycephalum are outlined in table 1. Much work on the cell cycle has been conducted using P. polycephalum because of the advantages of its natural mitotic synchrony, first recognised by Howard (1932) and which has been shown to occur in plasmodia as large as 20 cm² (Guttes et al, 1961). As all the nuclei divide synchronously in the absence of cell division in P. polycephalum this offers an opportunity to study nuclear division in a system which requires no artificial synchronisation.

Table 1

Stages of mitosis in *P. polycephalum*

Alcohol fixed smears from plasmodia growing in petri dishes at 22°C were photographed through a phase-contrast microscope to give the times indicated below.

Stage	Duration (min)	Events
Interphase	480	Chromosomes not visible, nucleolus distinct
Early prophase	15-20	Chromosomes appear. Centrioles move apart.
Prophase	5	Spindle organised. Chromosomes seen as 2 chromatids at equator. Nucleolus not visible.
Metaphase	7	Centromeres attach to spindle fibre at equator.
Anaphase	3	Centromeres divide. Chromatids move to opposite poles.
Telophase	5	Chromosomes less distinct.
Reconstruction	75	
Interphase	480	

from Mohberg & Rusch (1969)

Since single cells are too small to allow use of normal biochemical procedures, workers in other fields have had to develop methods with which to synchronise cells such that biochemical analysis could be undertaken. These methods may involve selecting cells at a specific stage in the cell cycle, ie selection synchrony. The earliest successful use of selection synchrony was by Maruyama and Yanagita (1956) who used fractional filtration and fractional sedimentation to select Escherichia coli cells at a specific stage in the cell cycle. Alternatively, workers use induction synchrony where, for example, DNA replication inhibitors or mitotic inhibitors are used to arrest cells at certain stages of the cell cycle thereby "lining the cells up" at a particular stage before allowing growth to resume again. In prokaryotes it is possible to use induction synchrony by selecting temperature-sensitive mutants which are arrested for example at the start of DNA replication when transferred to the non-permissive temperature, thereby lining all the cells up at the start of DNA replication. This has been successfully employed with Bacillus subtilis (Mandelstam & Higgs, 1974). The main criticism of these methods is that by interfering with the normal cell cycle the physiology of the cells obtained may not be normal. As an alternative, other workers looked for naturally occurring synchronous material, eg sea urchin eggs, insect eggs or giant amoebae. Mitotic synchrony in some myxomycetes was recognised as long ago as the end of the last century (Lister, 1893; Harper, 1900). These organisms and in particular P. polycephalum have proved particularly attractive since they combine mitotic synchrony with giant size. This provides large quantities of material in defined stages of the cell cycle which can be used for investigation of the mechanisms controlling cell growth and mitosis.

ii) Mechanisms controlling mitotic synchrony in *P. polycephalum*

As mitoses in the plasmodia of *P. polycephalum* are so highly synchronous, the underlying mechanism has attracted much attention. Basically there were two possibilities, either each nucleus had its own biological clock or a cytoplasmic factor was produced, the concentration of which determined when mitosis occurred. The results of a considerable number of experiments indicate that the latter is the case, ie a cytoplasmic factor is involved.

It was first noticed that when microplasmodia were fused to form a surface plasmodium, the time of the first mitosis did not vary by more than 5 min between all the nuclei in the plasmodium (Guttes et al, 1961). Later it was demonstrated that greater than 99% of the nuclei of this newly formed plasmodium underwent mitosis at the same time after fusion (Guttes & Guttes, 1964). Fusing two plasmodia which were predetermined as being at different stages of the cell cycle also resulted in a plasmodium which exhibited synchronous mitosis (Rusch et al, 1966). The time at which mitosis occurred in these plasmodia indicated that those nuclei farthest away from mitosis prior to fusion had been accelerated towards mitosis, whilst those nearer to mitosis had been delayed. It was concluded that a factor responsible for initiating mitosis was formed in the cytoplasm, increasing in amount during interphase and reaching a maximum prior to mitosis. Heat shock delays mitosis (Brewer & Rusch, 1968); it also delays the peak of thymidine kinase activity which occurs shortly before mitosis (Wright & Tollon, 1978). It would appear therefore that the factor responsible for initiating mitosis is heat sensitive. Oppenheim and Katzir (1971) advanced the onset of mitosis using an extract of late G2 plasmodia, the activity of which again was shown to be heat labile and was also

non-dialyzable. Nuclei do however reach a stage when the time of mitosis can no longer be altered; nuclei on the point of entering mitosis are unaffected by the introduction of fresh cytoplasm (Chin et al, 1972). Only one report of acceleration of mitosis using a specific protein has been made; Bradbury et al (1974a,b) accelerated mitosis by injecting H1 histone phosphokinase into the cytoplasm of plasmodia. They proposed that this enzyme triggered an increase in the phosphate content of the H1 histone which, it had been shown, occurred naturally before mitosis. Later the same group of workers demonstrated that the enzyme was not newly synthesised during G2 but was already present and only activated during G2 (Mitchelson et al, 1978). Other workers have confirmed that phosphate does accumulate in H1 histone shortly before mitosis and suggest that it may be related to chromosome condensation (Fischer & Laemmli, 1980). However, contrary to Bradbury et al (1974a,b) these workers observed no dephosphorylation after mitosis and hence they concluded that dephosphorylation is not essential for post mitotic de-condensation of chromosomes.

Immunological methods have also been used to demonstrate that late G2 cytoplasm contains higher levels of mitotically active substance than S phase cytoplasm (Blessing & Lempp, 1978). These workers presumed that the antigenic pattern of late G2 and S phase plasmodia would differ only with regard to their unequal quantity of mitotic initiator factor. Hence by treating a cell free extract of late G2 plasmodia with antisera prepared against early S phase plasmodia, they presumed they would enrich the extract for the factor which initiates mitosis. This antibody-treated late G2 extract caused an acceleration of the time of mitosis if introduced into G2 plasmodia. It can

therefore be concluded that synchronous mitosis of nuclei in plasmodia of P. polycephalum is controlled by a cytoplasmic factor. It is generally thought that this factor is not synthesised during S phase, but during G2 it is synthesized at a rate proportional to the cell size and when enough has accumulated mitosis is initiated (Tyson et al, 1979). This factor is most probably a protein; since plasmodia exposed to short pulses of cycloheximide treatment during G2 show a delay in mitosis (Scheffey & Wille, 1978).

Plasmodia of P. polycephalum have been used to demonstrate that U.V. light affects initiation of mitosis, since plasmodia exposed to U.V. light in S phase show a delay in initiation of mitosis (Guttes & Guttes, 1968a). They have also been used to demonstrate that low intensity electromagnetic fields can affect biological systems since these also can delay onset of mitosis (McCorquodale & Guttes, 1977; Marron et al, 1975).

iii) Dependence of mitosis on DNA replication

DNA replication must be completed before mitosis can be initiated (Sachsenmaier & Rusch, 1964). This was confirmed by demonstrating that DNA replication interrupted by U.V. light affected the onset of the next mitosis (Devi et al, 1968). Early experiments had suggested that a set G2 time of several hours was required after DNA replication before mitosis could take place (Sachsenmaier & Rusch, 1964), but plasmodial fusion experiments clearly demonstrated that under certain experimental conditions the G2 period could be substantially reduced (Guttes & Guttes, 1964; Chin et al, 1972). After completion of DNA replication it would therefore seem that mitosis can be initiated at any time but, under normal conditions, a G2 period is required for the transcription and synthesis of substances essential

for mitosis and growth of the plasmodium.

c) DNA replication in *P. polycephalum*

The time of DNA replication in the cell cycle of *P. polycephalum* was first demonstrated by Nygaard et al (1960) who reported that nuclear DNA is replicated immediately after mitosis; that is, there is no G1 phase in the cell cycle of *P. polycephalum*. Braun et al (1965) confirmed this and observed that the maximum rate of DNA synthesis was reached within 5 min of the uncoiling of telophase chromosomes. These experiments were conducted by transferring plasmodia to medium containing tritiated thymidine for 10 min pulses and subsequently immersing in TCA-acetone and measuring the incorporation of radioactivity into the acid-insoluble fraction. After mitosis, DNA replication continues for 2 to 3 h of the 8 to 10 h cell cycle (Nygaard et al, 1960; Braun et al, 1965). DNA synthesis was shown to be initiated simultaneously in 99% of the plasmodial nuclei (Braun et al, 1965), hence the synchrony of cell cycle events was maintained even at the level of DNA replication. Furthermore utilising the synchrony of DNA replication in *P. polycephalum* it was demonstrated that small regions of DNA replicated at specific times in S were replicated at the same relative time during the S phase of the next cell cycle (Braun et al, 1965; Braun & Wili, 1969). These experiments were conducted by labelling DNA with $[^3\text{H}]$ thymidine, $[^{14}\text{C}]$ thymidine and BUdR as a density marker, to differentiate between regions synthesised at specific times. Eukaryotes differ from prokaryotes in that they do not replicate their DNA from a single starting point. Replication occurs at numerous points and proceeds bidirectionally, hence it does not occur in sequence along a chromosome, but the above experiments demonstrated that it was sequential with respect to time. Evidence was therefore available that DNA synthesis was synchronous at the molecular level.

As mentioned previously, early experiments were conducted using pulse labelling techniques with tritiated thymidine. One criticism of these techniques is that thymidine is subject to catabolism in plasmodia such that radioactive label may be associated with products other than DNA. More recently isotope dilution methods, which overcome the problem of catabolism, have been used to confirm that DNA synthesis does take place immediately after mitosis, with no apparent G1 phase (Turnock, 1979). The isotope dilution method involves giving microplasmodia a pulse of radioactivity and then allowing time for the radioactivity to be incorporated into stable macromolecules. A surface plasmodium is then formed from the microplasmodia in the absence of further label, the specific activity of the macromolecule involved will decrease as synthesis takes place. Accumulation of the macromolecule is inversely proportional to the specific activity. Thus it has been shown that although the majority of the DNA is replicated immediately after mitosis, some small amount of replication continues in G2 phase (Hall et al, 1975; Hall & Turnock, 1976). Some of this replication during G2 has been demonstrated to be chromosomal (Hall & Turnock, 1976) but this seems mainly to be due to aberrant nuclei, the majority of the DNA replicated during G2 seems to be genes coding for rRNA. This rDNA, as in other eukaryotes, is mainly found in the nucleolus (Hall et al, 1975) and is synthesized throughout the cell cycle with the exception of the first hour after mitosis (Hall et al, 1975; Turnock, 1979).

DNA replication appeared to be dependent on protein synthesis (Cummins & Rusch, 1966), but addition of cycloheximide at various times during S still allowed a certain amount of DNA to be replicated. The term "a round of DNA replication" was suggested, with S consisting of 3 to 5 rounds of DNA replication (Cummins & Rusch, 1966; Cummins, 1968).

Later evidence suggested the existence of some ten replicative units (Muldoon et al, 1971). It was proposed that each round of replication required to be initiated by an initiator protein and that those proteins necessary for later rounds of replication could not be synthesised until the previous period of replication was complete. Mitosis would initiate the first round but later rounds required protein synthesis (Cummins & Rusch, 1966; Cummins, 1968). This idea has not been expanded much since then although cycloheximide has been shown to completely inhibit DNA strand elongation within 15 min of addition (Evans et al, 1976). The effect of cycloheximide has been shown to be primarily an effect on protein synthesis with the effect on DNA synthesis being a result of failure of protein synthesis (Evans & Evans, 1980). DNA replication does, therefore, seem to be dependent on active protein synthesis. DNA replication is dependent on mitosis having been initiated, but not necessarily completed, since heat shock administered 15 to 20 min before metaphase results in a 'pseudomitosis' in which normal mitotic events do not occur but DNA replication proceeds normally (Brewer & Rusch, 1968). Furthermore if G2 nuclei are placed in S phase cytoplasm they cannot be induced to initiate another round of DNA replication (Guttes & Guttes, 1968b). Mitosis may in some way render the chromosomes structurally competent for DNA replication. Chromosomal DNA replication, therefore, takes place immediately after mitosis with no intervening G1 period. Mitosis must have been initiated but not necessarily completed and protein synthesis is required for DNA replication to take place.

d) Transcription

Nygaard et al (1960) investigated RNA synthesis during the cell cycle and noted that it was greatly reduced during mitosis. Later pulse-labelling studies indicated that the pattern of total RNA synthesis

was biphasic, with peaks at 2 to 3 h after and 2 to 3 h before mitosis and minimum synthesis during mitosis and at a point in G2 (Mittermayer et al, 1964; Mittermayer et al, 1965; Braun et al, 1966; Kessler, 1967). This biphasic pattern of synthesis was also apparent in isolated nuclei and nucleoli (Mittermayer et al, 1966). Apparently transcription is switched off during mitosis and restarted at about 5 min after anaphase, at about the same time as DNA synthesis (Kessler, 1967). More recently it has been confirmed by isotope dilution methods that both rRNA and tRNA synthesis cease during mitosis (Hall & Turnock, 1976; Fink & Turnock, 1977).

Attempts have been made to determine whether the RNAs being transcribed in the two peaks apparent in pulse labelling experiments are different. That is, are there any qualitative changes in the RNA molecules being transcribed at the different points in the cell cycle. The premitotic and postmitotic periods of transcription have been shown to be different in their sensitivity to actinomycin D (Mittermayer et al, 1965). The RNA being transcribed in the first peak after mitosis has been shown to be DNA like, possibly hnRNA (heterogenous nuclear RNA, high molecular weight precursor to mRNA), whilst the second peak consists of predominantly rRNA (Grant, 1972). Nuclear transcription is at a maximum 2 to 3 h after mitosis and thereafter drops to a low level, whilst nucleolar rRNA synthesis is high throughout interphase rising to a peak 2 to 3 h before mitosis (Grant, 1972). This is consistent with data from Hall & Turnock (1976) where rRNA synthesis was continuous throughout interphase with the rate of synthesis increasing 5 to 6 fold during the course of interphase. It would therefore seem that the type and quantity of RNA transcribed during the cell cycle is closely controlled. By blocking transcription with actinomycin C, to which P. polycephalum was found to be more sensitive, it was estimated that

the last mRNA necessary for mitosis was synthesised at about 1 h before telophase by Sachsenmaier et al (1967) and 14 min before metaphase by Cummins & Rusch (1967).

Transcription therefore generally stops during mitosis and a change in the pattern of transcription is apparent when it restarts. The type and quantity of RNA transcribed throughout the cell cycle is under quite definite control and it is possible that mitosis provides a means of realigning the machinery of transcription at the start of the cell cycle.

e) Protein synthesis

Cycloheximide completely blocks the ensuing mitosis if it is added at any time prior to late prophase in the cell cycle. Consequently the last protein necessary for mitosis is synthesised very shortly before that mitosis (Cummins et al, 1965). All of the necessary structural proteins for mitosis are available before nucleolus dissolution in prophase (Cummins et al, 1966). Protein synthesis is continuous throughout the cell cycle (Mittermayer et al, 1966; Birch & Turnock, 1977) but its rate increases during the course of G2 (Birch & Turnock, 1977). Although overall protein synthesis is continuous, examination of uptake of lysine by pulse-labelling experiments revealed a biphasic pattern of incorporation into protein (Mittermayer et al, 1966) and consequently, control of protein synthesis during the cell cycle may be more complex than is first apparent. Examination of the number of polysomes present at various times in the cell cycle revealed no change associated with mitosis, which might have been indicative of certain functions ceasing during mitosis and starting afresh at the start of the next cell cycle (Mittermayer et al, 1966; Birch & Turnock, 1977).

Studies on P. polycephalum have revealed that a complex interaction of events occurs during interphase, allowing an orderly progression through the cell cycle. Further work on Physarum may help determine more exactly what forces control that progression through an orderly series of events.

3. Sporulation of P. polycephalum

a) Laboratory methods for obtaining sporulation

Sporulation is an essential part of the life cycle of P. polycephalum as it leads to genetic recombination during meiosis and to the propagation of the organism by spores. When plasmodia are starved, their appearance changes; they migrate, extending thin veins of protoplasm with fan-like extensions at their tips. If while migrating the plasmodium finds nutrient, then locomotion stops and it will resume growth. If however, a starved plasmodium does not find nutrient and is exposed to light then it will sporulate.

Howard (1931a,b) managed to cultivate the organism on rolled oat agar and followed the development of the plasmodia to form spores. This was mainly a morphological study but he did comment that presence or absence of nutrition apparently had no bearing on whether sporulation occurred or not. Seifriz and Russell (1936) considered the effect of 9 environmental factors on sporulation, including nutrition and light, and discounted them all as of little importance. At that time the most popular idea was that P. polycephalum had an intrinsic growth rhythm which determined when a plasmodium would sporulate regardless of environment. This reasoning was first disputed by Camp (1937) and again by Gray (1938). These workers proved, respectively, that sporulation could be delayed by addition of fresh nutrient and that

pigmented myxomycetes required light if sporulation was to occur. Gray (1938) also demonstrated that although light was necessary for sporulation, it was inhibitory to plasmodial growth. Therefore, by 1938 it had been established that fed plasmodia grew best in the dark whilst starved plasmodia required some amount of light to induce sporulation. As with all aspects of research on P. polycephalum more detailed investigation of sporulation was not possible until pure cultures of the organism were available.

In a follow up to their report of growth of plasmodia on soluble medium in 1961, Daniel and Rusch (1962a) published a method of obtaining sporulation in pure culture. Niacin they reported was an absolute requirement for sporulation (Daniel & Rusch, 1962b). Their method involved harvesting microplasmodia from shaken submerged culture and spreading them on a filter paper supported by glass beads in a petri dish. After allowing 4 to 5 h for the microplasmodia to fuse and form a plasmodium the filter paper was underlaid with a salts medium containing niacin. These plates were incubated in the dark for 4 days after which 2 h illumination produced sporulation within 16 h. Sporulation was obtained on a medium containing all the inorganic constituents of the growth medium plus niacin, as mentioned previously, as an absolute requirement; the optimal pH for sporulation was 5. Following this report, most workers have used this or similar methods to study sporulation of P. polycephalum (Table 2). In general, a fusion time of 2 to 6 h, starvation time of 4 to 5 days followed by 2 to 4 h illumination were used to obtain sporulation.

b) Factors affecting sporulation

Once a method of producing sporulation of Physarum on defined medium had been described more detailed consideration of the biochemical

Table 2

Summary of conditions used to obtain sporulation

Author & date	Time of fusion (h)	Time of starvation (d)	Time of illumination (h)
Guttes <u>et al</u> (1961)	2	4 to 5	2
Daniel & Rusch (1962a)	4 to 5	4	2
Daniel & Rusch (1962b)	4 to 6	4 to 5	2
Daniel & Baldwin (1964)	5 to 6	4	2
Daniel (1966)	6	4	4
Sauer <u>et al</u> (1969a)	4 to 6	4 to 5	4
Wilkins & Reynolds (1979)	1 to 3	3	4

and nutritional factors involved became possible. The first factor to be considered was the state of the microplasmodia prior to harvest. It was quite readily demonstrated that the optimal age of the microplasmodia for harvesting, if sporulation was subsequently to occur, was characterized by maximum growth of the microplasmodia (Daniel & Rusch, 1962a; Daniel & Baldwin, 1964). The volume of microplasmodia harvested appeared to have no effect on sporulation (Daniel & Rusch, 1962a).

The next point to be established was the minimum length of starvation after which illumination would trigger sporulation; this was placed at 4 days since earlier illumination gave a much lower percentage sporulation (Daniel & Rusch, 1962a,b). Furthermore niacin analogues which competitively inhibited sporulation only did so until the fourth day of starvation (Daniel & Rusch, 1962b). Cultures were therefore illuminated after 4 days starvation. More recently however, it has been suggested that sporulation competence can develop earlier than 4 days (Wilkins & Reynolds, 1979). These workers stated that development of sporulation competence was a function of both the duration of starvation and the ratio of plasmodial mass to medium volume (Wilkins & Reynolds, 1979). They proposed that some interaction between the plasmodia and the medium occurred such that larger plasmodia became competent to sporulate earlier than smaller plasmodia on a given volume of medium. They suggested that plasmodia released a substance into the medium the level of which determined when a plasmodium became competent, and since larger plasmodia would release more of this substance they sporulated earlier (Wilkins & Reynolds, 1979).

On the whole, however, cultures have been allowed 4 days incubation in the dark before illumination. It was very quickly realised that plasmodia were not committed to sporulation until after they had

been illuminated since addition of growth medium or glucose prevented sporulation if added prior to or during, but not after illumination (Daniel & Rusch, 1962a; Daniel & Baldwin, 1964). Later it was deduced using similar methods that plasmodia were not in fact committed to sporulation until some 3 h after illumination (Sauer et al, 1969a). Daniel (1966) suggested that since glucose only prevented sporulation when added before or during illumination then light might interfere with either glucose uptake or metabolism in this organism. Uptake of glucose has been shown to decrease during illumination but did not decrease further in the hours after illumination (Sauer et al, 1969a). Light has been shown to inhibit glucose metabolism, but not uptake in P. flavicomum (Lynch & Henney, 1973) whereas in Aspergillus ornatus both uptake and metabolism are inhibited (Hill, 1976). Since light inhibits growth and is necessary for induction of sporulation it is possible that these effects may be alternative expressions of the same photometabolism as it occurs in different metabolic environments. That is, stopping glucose metabolism inhibits growth, but in starvation conditions it commits the plasmodium to sporulation.

Since light has such pronounced effects on the plasmodia of P. polycephalum it has been presumed that the complex of yellow pigments they contain act as a photoreceptor and some evidence has been presented to support this theory (Gray, 1953; Daniel & Rusch, 1962a). More recently one group of workers claimed to have purified the pigment and demonstrated that injection of illuminated pigment into unilluminated starved plasmodia caused sporulation to occur (Wormington & Weaver, 1976). These workers had also previously reported that the requirement for illumination could be completely circumvented by microinjection of small amounts of salts solution into starved plasmodia (Wormington et al, 1975), although they were unsure whether sporulation was being induced

by the same or a distinct pathway. A mutant of P. polycephalum which forms white plasmodia has been isolated and is reported to sporulate just as well as the yellow plasmodia (Anderson, 1977); it is possible however that it is not defective in all the pigments which make up the yellow complex. It remains possible that light induces sporulation by some other mechanism; Daniel (1966) reported that light inhibits mitochondrial respiration in P. polycephalum. Light has also been shown to cause an increase in intramitochondrial granules, which seem to act as repositories for calcium, an increase in movement of calcium into mitochondria has also been observed (Nicholls, 1972; Daniel & Jarlfors, 1972). Cyclic uptake and release of calcium has also been shown to occur during the cell cycle (Holmes & Stewart, 1977).

Consequently it is uncertain whether a photoreceptor system or a mitochondrial energy change or some other as yet unknown mechanism is involved in the photometabolism which either induces sporulation or inhibits growth depending on the nutritional state of the plasmodium.

c) Biochemical changes during sporulation

Only one report concerning biochemical events during the course of starvation and morphogenesis has been published. Sauer et al (1969a) reported that sporulation of P. polycephalum required continuous protein synthesis, at least one DNA replication and mitosis before illumination and RNA synthesis until 3 h after illumination. Other workers have shown changes in protein pattern and RNA hybridisation pattern during sporulation (Zeldin & Ward, 1963; Jockusch et al, 1970; Sauer et al, 1969b) and the importance of these observations with respect to sporulation as a model of cellular differentiation will be discussed later. It would seem, however, that a change in transcription does

take place during sporulation but how this is controlled is uncertain. Whether mitosis and DNA replication during starvation are absolute requirements has still to be confirmed.

The study of sporulation of P. polycephalum is useful in that it may be able to contribute to our general understanding of eukaryotic cellular differentiation and morphogenesis. Studies of this nature are often hampered by lack of material clearly representative of the various stages of differentiation. The fact that sporulation occurs synchronously throughout a plasmodium and that the stages of growth and sporulation are distinct and separate make it an ideal organism in which to study differentiation. Before considering in more detail what might be gained from investigating sporulation of P. polycephalum it is worth considering what other simple eukaryotic models have contributed to our knowledge of cellular differentiation.

4. Cellular differentiation

a) Differentiation and the cell cycle

In prokaryotes, if cell division is occurring more rapidly than the time required to replicate the chromosome, then a second round of replication is initiated prior to completion of the first to ensure complete replication of DNA before each cell division. This situation is not possible in eukaryotic cells since cell division must be preceded by the orderly separation of the two sets of daughter chromosomes in meiosis or mitosis during which DNA replication cannot occur. The cell cycle of eukaryotes must therefore be very precisely regulated; the cell cycle of Physarum has already been discussed. The next point to be considered is whether the controls which regulate the cell cycle play a part in the control of cell differentiation. In the past it was

generally accepted that growth and differentiation were mutually exclusive phenomena, such that a cell could either undergo mitotic cell division or differentiate. Once a cell had been determined to differentiate, no more mitotic cell divisions would take place. This is now known not necessarily to be true, since mitosis occurs in differentiating cells of some organisms (Stockdale & Topper, 1966; Shininger, 1975). What is more important is whether these mitotic events play a role in the differentiation of these cells. That is, can cells in the interphase of the cell cycle alter their genetic expression directly or do they require to continue through the cell cycle and mitosis, altering their genetic expression after mitosis.

b) Importance of mitosis in differentiation

i) Dictyostelium discoideum

The life cycle of the cellular slime mould D. discoideum consists of two phases which have always been regarded as being completely separate stages. During the vegetative stage amoebae feed, grow and undergo mitotic cell division. Under starvation conditions the amoebae aggregate to form a grex which is capable of migrating to find nutrient, if the grex fails to find nutrient, then it differentiates to form a fruiting body (Loomis, 1975). Because development occurred in the absence of nutrient it was assumed that cell division was completely uncoupled from differentiation (Raper, 1941). Later it was shown that mitotic cell divisions could take place during differentiation, but development could occur without mitosis having occurred (Bonner & Frascella, 1952; Wilson, 1953; Ross, 1960). Sussman and Sussman (1960) used a mutant of D. discoideum which forms tiny aggregates during differentiation, they were therefore able to count the numbers of cells

present at all stages of development. Once again it was concluded that normal cell differentiation was not dependent on cell division.

One criticism of earlier experiments where mitosis was observed (Bonner & Frascella, 1952; Wilson, 1953; Ross, 1960) was that continued growth and cell division during experiments may have been due to the bacteria, which amoebae are fed on, remaining associated with the amoebae after transfer to starvation conditions, resulting in incomplete starvation. This was overcome when experiments were conducted using axenically grown amoebae (Zada-Hames & Ashworth, 1978). With these cultures it was demonstrated that there were two distinct periods of mitotic activity during development and that nuclear DNA replication also took place. These two peaks of activity were always associated with the same stages of development, the first began at the onset of development and ended at the beginning of aggregation, the second period reached a maximum at the grex stage of development (Zada-Hames & Ashworth, 1978). These results suggest that mitotic cell division may be an integral part of the developmental process. The fact that cells taken from stationary phase which have ceased cell division were shown to re-enter the cell division cycle whilst no nutrient was available also implies that progress through the cell cycle is important in differentiation of D. discoideum.

With an inhibitor of DNA synthesis (5-fluoro-2'-deoxyuridine) and of mitosis (colchicine) it appeared that differentiation was dependent on these events since these inhibitors prevented differentiation (Cappuccinelli & Ashworth, 1976). More recently however work conducted with different inhibitors of mitosis has led to the renewed claim that differentiation of D. discoideum can occur without mitosis. First Cappuccinelli et al (1979) used nocodazole as a mitotic inhibitor;

nocodazole has been shown to be more effective in lower eukaryotes than colchicine and consequently can be employed at much lower concentrations (see section on biochemical changes in sporulation). Nocodazole was demonstrated to inhibit mitosis but differentiation could still take place in its presence hence it was concluded that mitosis was not necessary for differentiation to occur (Cappuccinelli et al, 1979). Second using yet another mitotic inhibitor isopropyl N-(3 chlorophenyl) carbamate (CIPC) it was once again claimed that mitosis was not required (White et al, 1981). Furthermore these authors isolated mutants which were resistant to the effects of CIPC during growth but remained sensitive during development. This they suggested indicated that mitosis was not the main target for the drug during development since they had demonstrated that during growth all the cells were arrested in mitosis indicating that mitosis was the main target during growth (White et al, 1981). They tentatively suggested that during the developmental phase cytoplasmic microtubules might play some role and it is this function which is being affected since CIPC, like colchicine and nocodazole, has its effect on the function of microtubules. This possibility will be discussed later in ^{the} section on biochemical changes in sporulation. Consequently it remains unclear whether mitosis or possibly some other event dependent on cytoplasmic microtubules is required for differentiation of D. discoideum.

ii) Saccharomyces cerevisiae

In S. cerevisiae haploid cells grow vegetatively by mitotic division; zygotes are formed by fusion of cells of opposite mating types. Meiosis and sporulation can be induced by changing the medium, usually to a nitrogen-free medium (Pinon, 1977; Delavier-Klutchko & Durieu-Trautman, 1978). Sporulation is dependent on continuous protein synthesis

(Magee & Hopper, 1974). The main advantages of working with S. cerevisiae are its ease of culture and the fact that it has a well documented genetic system (Mortimer & Hawthorne, 1969). The one major disadvantage is the necessity of synchronisation of cultures before conducting experiments. The main point of interest from work on S. cerevisiae is how genetic knowledge of an organism can assist investigation. Hartwell et al (1970) isolated temperature sensitive cell division cycle mutants (cdc) of S. cerevisiae. Using these mutants knowledge of the cell cycle of S. cerevisiae has been considerably advanced (Hartwell, 1974, 1976). Genetic analysis of 148 of these mutants revealed that they contained mutations in 32 cdc genes, blocked at various stages of the cell cycle, many represented by more than one allele (Hartwell et al, 1973). Hartwell et al (1974) summarized the behaviour of 20 cdc mutants. Using these cdc mutants an attempt was made to determine whether only cells at a particular stage of the cell cycle could differentiate. Hirschberg & Simchen (1977) concluded that only cells blocked in early G1 could go directly into differentiation. All other mutants blocked later in the cell cycle had to first complete the cycle and go through mitosis before being able to differentiate (Hirschberg & Simchen, 1977). That is, only cells in G1 can start differentiation (Simchen, 1978). This has led to the concept of 'start' in the cell cycle of S. cerevisiae (Hartwell, 1974; Nurse, 1981). This is the earliest known gene-controlled event in the cell cycle which occurs early in G1. Once cells have passed 'start' they are committed to the mitotic cell cycle. Only cells of a certain mass are able to complete 'start' (Nurse, 1981) and hence on nutrient deprivation progress through the cell cycle is stopped, and cells accumulate at 'start' before differentiation occurs. A similar start mechanism has also been shown to exist in Schizosaccharomyces pombe (Nurse & Bisset, 1981).

Consequently using the large range of cdc mutants in S. cerevisiae it has been possible to determine an exact point in early G1 where cells make the decision to either differentiate or continue progression through the cell cycle. Once past this point cells must continue through S, G2 and mitosis before differentiation can occur.

iii) Other model systems

From the above consideration of two simple eukaryotic models it would seem possible that some requirement for cell cycle events during differentiation may exist. Similar evidence has been obtained from more complex eukaryotic models eg. terminal differentiation of the cells of the mammary gland in mice only occurs after a hormone-induced cell division (Stockdale & Topper, 1966; Vonderhaar & Topper, 1974); continued progress through the cell cycle is required for formation of tracheary elements in some plants (Shininger, 1975).

It is possible in the examples considered that cell cycle regulation may have some control over the process of differentiation. At one point the possibility of two types of cell cycle was considered, one being proliferative and the other involved in differentiation (Rutter et al, 1973), however this idea has been dismissed for lack of evidence. It appears more likely that control of regulation of progression through the cell cycle also plays a part in differentiation. As mentioned previously, cells not destined for immediate division are generally arrested in G1 phase, and the fact that differentiated cells frequently cease dividing has led to the belief that these cells are in a specialised G1 phase termed G0.

In addition to the start point in the cell cycle of S. cerevisiae a similar point has been described for mammalian cells; cells accumulate at this point in the cell cycle in conditions of minimum metabolism.

Once again this is a point in early G1 phase and has been called the restriction point (Pardee, 1974). It is thought possible that cells which are arrested at this point in G1 may be the equivalent of cells said to be in the specialised G1 or G0 phase previously mentioned (Pragnell et al, 1980). Furthermore ts mutants of Friend erythro-leukaemia cells have recently been isolated (Conkie et al, 1981). Using these it has been demonstrated that these cells also have a requirement for a cell cycle event in G1 to occur before they can differentiate and become capable of synthesising haemoglobin (Conkie et al, 1981). Consequently it may be possible that according to the environmental conditions during the previous cell cycle, cells either pass this restriction point or 'start' and enter another mitotic cell cycle or they accumulate at this point from which if necessary they can enter alternative pathways of development.

iv) Control of gene expression during differentiation

One of the earliest theories of differentiation was that it was brought about by a progressive loss of genes. That is, as a chromosome became more highly specialised so the cell became more specialised and the particular developmental pathway pursued was determined by which genes were lost. Since it is now believed that somatic cells in most organisms have identical sets of genes, differentiation must arise by mechanisms other than loss of genes.

Mammalian cells have sufficient DNA to code for thousands more proteins than are required for normal cell functions, consequently a considerable amount of DNA is never read into RNA. The fine structure of the gene and the interaction of protein molecules with it determine what genes are transcribed. During differentiation it could therefore be possible that some genes are differentially transcribed ie. read into RNA at a faster rate (Gurdon, 1973).

A considerable amount of work has been undertaken to identify the protein molecules which by their association with DNA might regulate which genes are preferentially transcribed. Consideration of protein molecules which naturally associate with DNA first led to the suggestion that histones might be involved in regulation, but these do not have the heterogeneity which would be required of regulator molecules. The histones are mainly involved in packaging DNA such that DNA connected with an octamer of histones has been shown to form the basic structural unit of the chromosome, the nucleosome (Felsenfeld, 1978). More recently attention has turned to the non-histone nuclear proteins as possible regulator molecules. These have been shown to vary between different species and even between tissues of one organism and consequently have the tissue and species specificity which would be required of regulator molecules. Paul (1971) suggested that the non-histone proteins worked in collaboration with the histones, which he suggested, played some generalised role in regulation of gene expression. Genes which are active in any particular tissue were thought to have an altered relationship with the histones. This altered relationship was brought about by interference by the gene specific non-histone proteins (Paul, 1971). Furthermore the complement of non-histone proteins in amoebae and plasmodia of P. polycephalum have been shown to be quite different (LeSturgeon et al, 1973), and significant changes in the complement of non-histone proteins have been observed during differentiation of P. polycephalum ie. during starvation (LeSturgeon et al, 1973; LeSturgeon et al, 1975; LeSturgeon, 1977). In fact Physarum was the first experimental system in which such changes were observed (LeSturgeon & Rusch, 1971). Similar changes have been shown to occur when microplasmodia are subjected to conditions of high density, microplasmodia

were centrifuged and the pellet allowed to stand (McAllister et al, 1977), suggesting a generalised mechanism for transition from active growth to non-proliferation. The non-histone proteins may therefore have a role in controlling gene expression during differentiation.

As well as consideration of changes in nuclear proteins changes in the cell cytoplasm also affect cell differentiation. This was demonstrated by nuclear transplant experiments where a nucleus from a specialised cell was transplanted into embryonic cytoplasm, the nucleus of this cell was destroyed. This resulted in development of a complete organism, demonstrating that the cytoplasm was able to 'switch' on genes which had been 'switched off' in the specialised cell (Gurdon, 1968). Consequently the cell cytoplasm also plays a role in control of gene expression. This led Gurdon (1973) to present a model of cell differentiation (Fig 2) which involved synthesis of cytoplasmic regulatory molecules which were able to associate with the nuclear material during mitosis thus changing gene expression in the cell after mitosis. It is possible that some mechanism of this sort allows cells to take stock of the nutritional state of the cell and determine whether the cell will become arrested in early G₁, ie G₀, or enter another cell cycle.

Consequently some control of gene expression is possible at the level of gene transcription ie. choosing which genes are transcribed at what times. However, more recently it has become apparent that this is not the only level at which control can be exerted. Unlike prokaryotic mRNA, eukaryotic mRNA is now known to be synthesised in the nucleus as part of a long transcriptional precursor unit (heterogenous nuclear RNA, hnRNA). This hnRNA is subject to various modifications before it arrives in the cytoplasm of the cell as mature mRNA to be translated into protein (Banerjee, 1980). The greatest problem in devising a

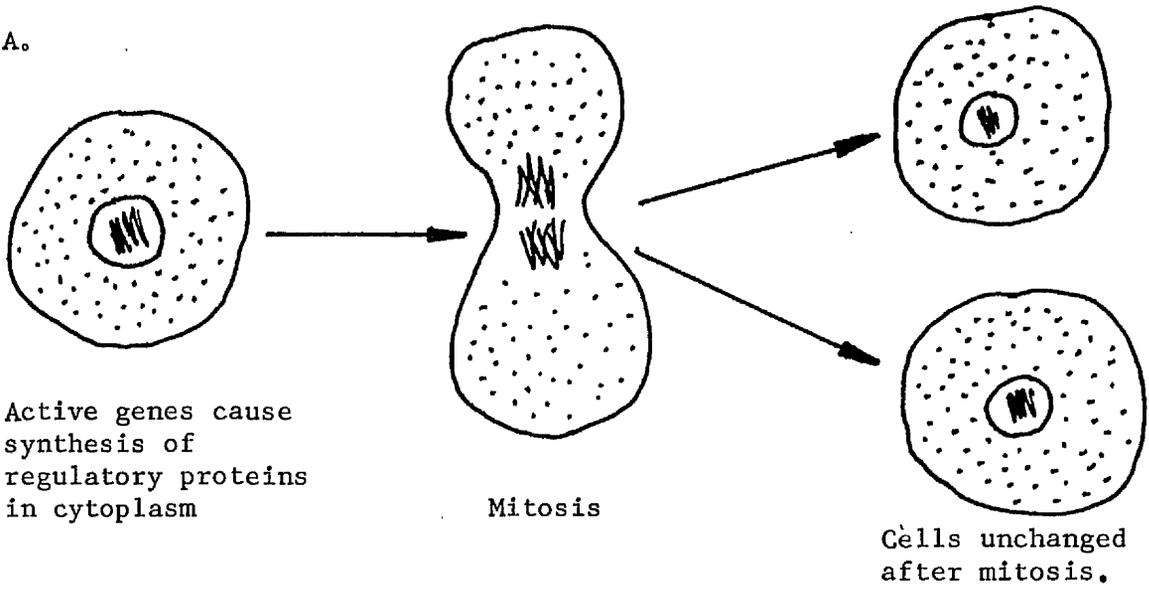
Figure 2

Model for reprogramming of gene activity at mitosis.

(adapted from Gurdon, 1973)

- A. Normal cell division
- B. Differentiation

A.

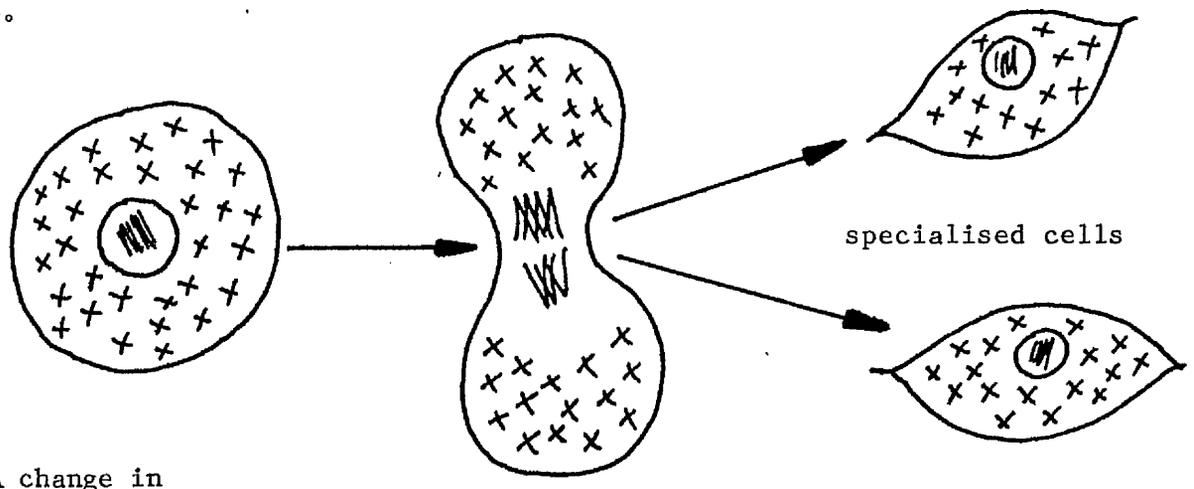


Active genes cause synthesis of regulatory proteins in cytoplasm

Mitosis

Cells unchanged after mitosis.

B.



A change in regulatory protein is stimulated.

Old regulatory proteins dissociate from chromosomes during mitosis and new proteins are associated during reconstruction.

specialised cells

Regulatory protein promote specialisation after association with chromosomes at mitosis.

method by which hnRNA gave rise to mRNA was the considerable difference in size, hnRNA units are much larger than mRNA units (Knowles & Wilks, 1980). This was overcome with the realisation that the genes for proteins often contain large inserts of non-coding material (introns) which are often larger than the coding sections of DNA (exons) (Doel, 1980). Maturation of mRNA therefore involves excision of the introns and ligation of those pieces of RNA which make up the coding portion of the message. Furthermore the RNA is subjected to various other processes of methylation, 5' terminal capping and 3' terminal polyadenylation before becoming mature mRNA in the cytoplasm (Banerjee, 1980). These steps must involve enzymes closely related with the RNA and it is likely that control of gene expression could be exerted at any of these points (Knowles & Wilks, 1980). Consequently as well as controlling which genes are transcribed, there can also be control at the post-transcriptional level. Some unspliced RNA precursors may be present in the nucleus but might never reach the cytoplasm as mature mRNA (Minty & Newmark, 1980). During differentiation therefore, cells may arise with similar nuclear RNA content but their cytoplasm may differ greatly in type and number of mRNAs available for translation into protein (Jacobs & Birnie, 1980) i.e. there is post-transcriptional control of the type and number of mature mRNA molecules reaching the cytoplasm. This has led to the idea of a cascade of controls of gene expression of which transcriptional control is the first and after which a series of controls are involved in maturation and transportation of mRNA to the cell cytoplasm (Birnie, 1980).

Only by examination and comparison of as many eukaryotic models as possible will it be possible to determine what factors are generally involved in eukaryotic differentiation. It may then be possible to

contribute something to our understanding of the nature of cancer cells which are basically cells which have failed to recognise the signal to differentiate. It may be that malignant cells are cells which fail to recognise their restriction point or start control which regulates their cell cycle and development (Pardee, 1974).

5. Sporulation in *P. polycephalum* as a model of cell differentiation

If it is taken that every non-mutational permanent variation in cell structure or behaviour is a result of a differentiative process (Gross, 1968), then sporulation of *P. polycephalum* is clearly an example of cellular differentiation. As a model for studying differentiation it has the advantage that differentiation occurs synchronously under environmental conditions which can be controlled by the worker. Hence after initiation of sporulation in the starved plasmodium by light, differentiation and morphogenesis take place synchronously throughout the plasmodium, and since this occurs even in very large plasmodia sufficient material for biochemical analysis is also available.

Problems which could be investigated using sporulation as a model would include the importance of mitosis and DNA replication in differentiation and whether progression through the cell cycle to a specific point is required for differentiation. In conjunction with biochemical investigations it would be useful to be able to analyse the process genetically, the advantages of this having been demonstrated by the work on the yeast cell cycle. With this in mind the current state of knowledge of these aspects of sporulation must be considered.

a) Biochemical changes associated with sporulation

Much work has been carried out on mitosis in the normal cell cycle of *P. polycephalum*, as was discussed earlier. On the other hand

very little information about mitosis and DNA replication during starvation is available. Guttess and Guttess (1961) claimed that plasmodia continued to undergo synchronous mitosis under starvation conditions but the interphase time was much longer at between 24 and 36 h. Mitosis they observed was immediately followed by DNA replication so that no G1 phase was apparent even in starvation (Guttess & Guttess, 1961). Sauer et al (1969a) claimed that at least one mitosis and DNA replication must occur in the starved plasmodium before illumination would induce sporulation. Mitosis immediately followed by DNA replication occurred about 4 hours before starved plasmodia were illuminated after 92 h starvation. This work was carried out by microscopic observation of mitosis, assay of incorporation of [³H]thymidine and use of inhibitors of DNA synthesis.

Two of the inhibitors of DNA synthesis used by Sauer et al (1969a) were 5' bromodeoxyuridine (BUdR) and hydroxyurea. Hydroxyurea inhibits the action of the enzyme ribonucleoside diphosphate reductase which is required for production of all four DNA purines and pyrimidines (Perlman & Rownd, 1975; Ockey & Allen, 1975). More recently it has been shown that in the yeast S. cerevisiae hydroxyurea inhibits joining of completed replicon units in vivo (Johnston, 1980). Since hydroxyurea is known to inhibit ribonucleoside reductase an effect on DNA polymerase at the level of precursor supply is suggested, as a result the drug may inhibit a polymerising activity responsible for filling the gaps left between adjacent completed replicons (Johnston, 1980). BUdR (an analogue of thymidine) has been used in several studies on differentiation since it appears to exert a greater inhibitory effect on differentiation than on growth (Rutter et al, 1973). How BUdR exerts its effect is not clearly understood as there have been conflicting reports stating that BUdR acts by being incorporated into

DNA (Guespin-Michel et al, 1976) or that it is not incorporated (Rogers et al, 1975), that BUdR has specific sites of action during development (Lough & Bischoff, 1976) or that there is no particular stage of development at which it acts (Kay et al, 1978). It seems that the effect of BUdR incorporation into DNA is on transcription, either the BUdR is incorporated into gene initiator sites and prevents initiation of transcription or incorporation of BUdR into DNA causes tighter binding of regulatory molecules so preventing transcription of those products necessary for differentiation. One report of its effect on D.discoideum suggests that the effect is the same in both growth and development, reducing transcription by about 20% (Monier et al, 1978). This reduction has no effect on growth but prevents development since transcription has already been greatly reduced by the starvation conditions required for development and a further 20% reduction completely blocks differentiation (Monier et al, 1978). Although the method of action of BUdR is not clearly understood it is possible that use of this analogue may help clarify some of the regulatory mechanisms involved in differentiation.

The most effective inhibitor of DNA synthesis is 1 β arabino-furanosyl cytosine (AraC). As well as inhibiting DNA replication, AraC can also induce chromosome breaks, maximum sensitivity with this respect being in the latter half of S and early G2 (Benedict et al, 1970). The main effect of AraC was thought to be on polymerase activity thus inhibiting elongation of DNA chains (Rashbaum & Cozzarelli, 1976). More recently however one worker using much lower concentrations of AraC, suggested that the primary effect was on initiation of new chains of synthesis without any immediate effect on chain elongation (Fridland, 1977).

Similar to inhibitors of DNA replication, inhibitors of

mitosis could be used to assess the importance of mitosis. The inhibitor of mitosis most frequently used in the past was colchicine, which completely inhibits the formation of microtubules from their protein subunits and hence disrupts the function of the spindle during mitosis. Colchicine has been reported to delay mitosis in plasmodia of P. polycephalum (Hagino et al, 1978) but more recently there have been several reports that colchicine has a much poorer effect on lower eukaryotes than it has on higher eukaryotes (Williams, 1980; Quinlan et al, 1980; Quinlan et al, 1981). With Polysphondylium pallidum, S. cerevisiae and D. discoideum the benzimidazole carbamate, nocodazole has been found to be more effective in preventing mitosis (Welker & Williams, 1980; Williams, 1980; Quinlan et al, 1980). Nocodazole has a similar mode of action as colchicine, interfering with the structure and function of microtubules (DeBrabander et al, 1976). Quinlan et al (1981) compared the effect of nocodazole and colchicine on assembly of purified microtubule proteins from P. polycephalum with their effect on similar proteins isolated from higher eukaryotic cells. Both drugs were capable of preventing assembly of subunits from the higher eukaryote but colchicine was much less effective against the Physarum microtubule proteins than nocodazole (Quinlan et al, 1981). Consequently it would seem that microtubule assembly in lower eukaryotes is less sensitive to colchicine than is assembly in higher eukaryotes and the difference in response to the drug between these organisms is not due to differential uptake or metabolism. Consequently of the two inhibitors, nocodazole would be the more useful in assessing the importance of mitosis during differentiation of P. polycephalum. Also since nocodazole can be used at much lower concentrations it is more likely to be specifically affecting mitosis, early work with colchicine was conducted

with very high concentrations of the drug which could possibly have been affecting functions other than assembly of microtubules.

One final point has to be considered when using these 'mitotic' inhibitors, that is, as mentioned previously, the possibility that they could also affect functions involving cytoplasmic microtubules. Cytoplasmic microtubules are found in a large number of cell types, their role being uncertain although they may be involved in cell morphology or movement. During growth of D. discoideum and P. polycephalum the main target for inhibitors of microtubule assembly would appear to be the function of the spindle during mitosis (Welker & Williams, 1980; Quinlan et al, 1981). The possibility that cytoplasmic microtubules might play some role in development cannot however be ruled out. This possibility should therefore also be considered when assessing results obtained from work employing these drugs during differentiation.

Some work on changes in RNA and protein synthesised during sporulation has been conducted. Interest in RNA is mainly in determining whether different RNAs are transcribed during differentiation. One report does suggest the existence of different RNA moieties in growing and starving cultures (Sauer et al, 1969b). Whilst studying the RNA content of plasmodia it was observed that P. polycephalum contains a polyphosphate, the concentration of which is inversely proportional to the content of RNA (Sauer et al, 1969c). The polyphosphate content has been shown to increase to a maximum prior to mitosis in the cell cycle and also increases during starvation (Goodman et al, 1969). This could be important with respect to the role of mitosis in differentiation.

Changes in protein pattern have been demonstrated to occur during sporulation by electrophoresis (Zeldin & Ward, 1963; Jockusch

et al, 1970). The major new proteins synthesised appear to be structural and can be extracted from mature cell walls. Also, as mentioned earlier, changes in non-histone protein content have also been observed during sporulation.

Using sporulation of P. polycephalum as a model of differentiation it should therefore be possible to help clarify the role of mitosis and DNA replication in differentiation.

b) Genetics of P. polycephalum

The earliest attempt at genetic analysis of P. polycephalum was in 1962 by Dee, who reported for the first time the existence of amoebae of different mating types such that only amoebae of opposite mating types could fuse and form plasmodia. At that time only two mating types designated + and - were discussed. Later work extended the number of mating types to 4 (Dee, 1966b) and then to 12 and 14 (Collins, 1975; Collins & Tang, 1977). These were found to be alleles of the one locus mt and only strains carrying different alleles of mt could cross to form plasmodia. The possibility of genetic analysis of P. polycephalum was considerably improved by the discovery in 1970 of a strain which formed plasmodia from amoebal clones ie. no crossing of amoebae was required (Wheals, 1970). This isolate designated C50 (Colonia 50) could cross with all other mating types as well as form plasmodia in clones (Wheals, 1970; Adler & Holt, 1974) and so it was deduced that this strain carried another allele of the mt locus designated mth. Later a strain was derived from C50 which produced plasmodia in clones with much higher efficiency - the CL strain (Colonia Leicester) (Cooke & Dee, 1975). More recently a second locus affecting mating between amoebae has been described. This was first called the rac gene (Dee, 1978) and it was reported that amoebae carrying different

mt alleles would cross more readily if they differed in their rac allele also (Dee, 1978). Youngman et al (1979) also described a second mating compatibility locus, and changed the terminology of the original mt locus. They called mt, matA and the new locus matB; matB is equivalent to the rac gene described by Dee (1978). Again it was reported that strains differing in both matA and matB cross more readily (Youngman et al, 1979). Dee (1978) described two rac alleles and Youngman et al (1979) described three matB alleles; a total of 13 alleles of matB have since been described (Kirouac-Brunet et al, 1981). MatA and matB are the terms now used for these two alleles, matA controls zygote differentiation into plasmodia (Adler & Holt, 1975; Kirouac-Brunet et al, 1981) and matB controls amoebal fusion (Youngman et al, 1979). Only those strains carrying different matB alleles will fuse efficiently and only those carrying different matA alleles will differentiate to form plasmodia.

When Wheals (1970) originally described the *Colonia* isolate of P. polycephalum he proposed that development of plasmodia was homothallic. That is two identical amoebæ fuse to form a diploid zygote which then forms a diploid plasmodium. Mohberg & Rusch (1971) measured the DNA content of nuclei at various stages of the life cycle of a normal strain which formed plasmodia from crossed amoebae. They concluded that amoebae were haploid and plasmodia diploid and this was confirmed later (Mohberg et al, 1973). Using similar methods on the CL strain Cooke & Dee (1974) demonstrated that plasmodia formed from amoebal clones were haploid. Mohberg (1977) presented similar data and it was concluded that the CL strain was haploid throughout its entire life cycle. This ruled out the homothallic method of plasmodial formation and the possibility that plasmodia arose apogamically from single amoebae was proposed (Cooke & Dee, 1974). This idea of an

apogamic life cycle was confirmed by the cinematographic study of events during plasmodia formation from amoebae by Anderson et al (1976).

The discovery of the CL strain considerably improved the chances of isolating plasmodial mutants since no crossing of amoebae was involved and consequently there was no necessity for laborious back-crossing and outcrossing of amoebae to produce plasmodia homozygous for a mutation. Whether plasmodia formation was homothallic as originally thought or apogamic as turned out to be the case had no effect on this interpretation of the advantages of the CL strain for genetic analysis since the plasmodia would have been homozygous in either case. It does however affect work on sporulation since a haploid plasmodium would be unable to undergo the meiosis which normally takes place during sporulation (Laane & Haugli, 1976). In fact the CL strain produces very few viable spores and it was proposed by Laane et al (1976) that these either arose from a few haploid nuclei which underwent a 'pseudomeiosis' in which the second meiotic division was suppressed, giving rise to viable cells, or viable spores were arising from normal meiosis which took place in the small number of diploid nuclei contained in the generally haploid plasmodium. The second possibility would seem to be involved since when CL plasmodia were heat shocked to increase the number of diploid nuclei present the number of viable spores rose dramatically (Laffler & Dove, 1977). During sporulation of the haploid CL strain of P. polycephalum it would therefore appear that the large number of haploid nuclei enter an aberrant pseudomeiosis which produces non-viable spores whilst the small number of viable spores obtained are from the small number of diploid nuclei present which undergo normal meiosis.

Genetic analysis of the Colonia isolate has led to the

description of several genetic markers in P. polycephalum, including resistance to cycloheximide (Dee, 1966a; Dee & Poulter, 1970; Haugli et al, 1972; Gorman & Dove, 1974), post-fusion incompatibility of plasmodia (Carlile, 1976), control of ability of amoebae to grow in axenic medium (McCullough et al, 1978) white plasmodia (Anderson, 1977) and several other markers. A review of those available at the time and the methods by which they were produced was published in 1973 (Dee, 1973). Much attention has been focussed on the transition from amoebae to plasmodia. After the discovery of the CL strain which formed plasmodia in amoebal clones several mutants of CL were described which were affected in their ability to form plasmodia, these were called apt or npf mutants (Wheals, 1973; Davidow & Holt, 1977; Shinnick & Holt, 1977; Anderson & Dee, 1977; Adler & Holt, 1977; Anderson, 1979; Honey et al, 1979). Mutations of the normal heterothallic strains which can form plasmodia in clones have also been reported; these are called gad mutations (Adler & Holt, 1977; Shinnick & Holt, 1977). This has led to the suggestion that matAh may be the result of a gad mutation from an original matA2 strain (Anderson, 1979). Thus normally, heterothallic strains form plasmodia after a cross between two amoebae, although mutations can arise (gad) which form plasmodia in clones, whilst the CL strain normally produces plasmodia apogamically, but mutants do exist which are less able to produce plasmodia in this way (apt and npf mutants).

Several workers have attempted to obtain temperature sensitive (ts) mutants which are affected in cell cycle events. Mutagenesis was normally produced by U.V. irradiation or use of the chemical mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The latter is an alkylating agent which produces transition type mutations through alkylation of

guanine which increases the frequency of guanine pairing with thymine instead of cytosine. After exposure to NTG or U.V. irradiation amoebae were screened for mutations. Enrichment methods to attempt to increase the number of ts mutants obtained were also used. Haugli and Dove (1972) attempted to enrich for mutants ts in DNA replication; they incubated amoebae in the presence of BUdR at the non-permissive temperature, where only wild types grew and incorporated BUdR. They then utilised the fact that BUdR substituted DNA is more sensitive to U.V. irradiation to kill these and enrich for the unaffected ts mutants. Gorman and Dove (1974) produced a 40 fold enrichment utilising the antibiotic netropsin which only kills growing cells. The main obstacle in this work was that mutants produced in amoebae frequently were not expressed in plasmodia. Several workers produced ts amoebae or ts plasmodia, but very few mutants ts in both were obtained (Wheals et al, 1976; Gingold et al, 1976; Sudberry et al, 1978; Del Castillo et al, 1978; Laffler et al, 1979). Nevertheless, several possible cell cycle mutations were identified; Wheals et al (1976) identified 3 possible blocks in mitosis, Gingold et al (1976) described a mutant with a DNA replication block, Del Castillo et al (1978) a further two possible mitotic blocks and Laffler et al (1979) a mutant thought to be blocked at a step late in G2 which is necessary for mitosis and DNA replication. By using a medium on which both amoebae and plasmodia could grow relatively well Burland & Dee (1979) reported that over 50% of the mutants isolated were ts in both amoebal and plasmodial phases. They reported isolation of two cell cycle mutants blocked in nuclear division which were obtained by cytological screening of amoebal cultures of ts mutants (Burland & Dee, 1980). It would therefore appear that obtaining mutants ts in both amoebae and plasmodia may be easier in future.

Once ts cell cycle mutants are readily available this should theoretically make possible the assessment of the importance of cell cycle events in sporulation of P. polycephalum since the mutations will be expressed in the haploid plasmodium of the CL strain during sporulation. However before genetic knowledge of P. polycephalum can be put to full use more knowledge of sporulation of the CL strain will be required. All early reports on sporulation of P. polycephalum were from diploid plasmodia (Daniel & Rusch, 1962a; Daniel, 1966; Sauer et al, 1969a; Sauer, 1973; Wilkins & Reynolds, 1979) whilst genetic analysis in the main is conducted with the CL strain which produces haploid plasmodia. Consequently, if genetic aspects of sporulation are to be investigated, it would be useful to investigate whether what is already known of sporulation of P. polycephalum applies to the CL strain. If the importance of mitosis and DNA replication during sporulation are also investigated in this strain then it may be possible to analyse sporulation both genetically by the use of ts cell cycle mutants (since mutants will be expressed in the haploid plasmodia) and biochemically in the one strain of P. polycephalum.

OBJECT OF RESEARCH

1. The central aim of this work was to explore the possible value of the haploid CL strain of P. polycephalum as a model for studying eukaryotic differentiation, in particular to determine whether there is a requirement for any cell cycle events prior to differentiation. It was also hoped to demonstrate the advantages of a system which can be analysed from both biochemical and genetical standpoints.

2. As a basis for this work it was first necessary to develop a system for obtaining sporulation of P. polycephalum CL in a reproducible manner. It was also hoped to determine when the plasmodia became committed to sporulation and on what this time of commitment was dependent.

3. When this had been achieved the main direction of the work was to determine:

- a) the importance of DNA replication and mitosis during differentiation. Inhibitors of DNA replication and mitosis would be used to assess the overall importance of these events, whilst uptake of [methyl-³H] thymidine into acid-insoluble material would be used to try and determine more exactly when DNA replication occurred during starvation.
- b) whether mutants which were unable to sporulate could be obtained and whether genetic analysis of the sporulation process might be feasible.

MATERIALS AND METHODS

1 Maintenance and culture of *P. polycephalum*

a) Strains of *P. polycephalum*

Physarum polycephalum CL was used for the majority of the work. This was obtained from T. Laffler, McArdle Laboratory for Cancer Research, Madison, Wisc., U.S.A. as a derivative of the original *Colonia Leicester* (CL) strain (Cooke and Dee, 1975) which sporulated consistently well. A CL culture and three other strains designated LU216, LU276 and LU862 were obtained as amoebal cultures from J. Dee, Genetics Dept., Leicester University. The genotypes of these strains are given in Table 3.

b) Culture of microplasmodia in liquid medium

For routine use the organism was maintained as a shaken suspension of microplasmodia. To subculture, 1.5 ml of a 72 h old culture of *P. polycephalum* was used to inoculate 20 ml of semi-defined medium (SDM) (Daniel and Baldwin, 1964) (Appendix 1) contained in a 250 ml dimpled conical flask. This was incubated in the dark at 26°C and shaken at 100 r.p.m. on a Gallenkamp orbital shaker.

c) Surface culture of plasmodia

Five ml of microplasmodia were collected in the late exponential phase of growth and harvested by centrifugation at 500 x g for 2 min at room temperature in a MSE super minor centrifuge. The microplasmodia were resuspended in a volume of sterile distilled water equal to the volume of the packed microplasmodia and were then pipetted in a circle onto a sterile Whatman 50 filter paper supported by a wire mesh in a 9 cm petri dish (Fig 3). The filter papers were sterilised by autoclaving for 15 min at 15 p.s.i. in glass petri dishes wrapped in aluminium foil, and were transferred to the wire mesh immediately before

Table 3

Genotypes of strains of *P. polycephalum*

<u>matA</u>	amoebal mating type, controls differentiation of zygotes into plasmodia (Adler & Holt, 1975; Cooke & Dee, 1975).
<u>rac</u>	second locus affecting amoebal mating now called <u>matB</u> , controls fusion of amoebae to form zygotes (Dee 1978, Youngman <u>et al</u> , 1979).
<u>fusa</u>	locus affecting plasmodial fusion. Only plasmodia with the same fusion genotype can fuse (Carlile & Dee, 1967; Poulter & Dee, 1968).
<u>npf</u>	locus affecting transition from amoebae to plasmodia. Revertant from the apogamic strain which cannot form plasmodia in clones as readily (Anderson & Dee, 1977).
<u>leu-1</u>	plasmodial requirement for leucine, <u>leu-1</u> is recessive to the wild type <u>leu</u> ⁺ (Cooke & Dee, 1975).
<u>whi-1</u>	white plasmodia; <u>whi-1</u> is recessive to <u>whi</u> ⁺ (yellow) (Anderson, 1977).
<u>eme</u>	resistance to emetine. (Dee, 1962)

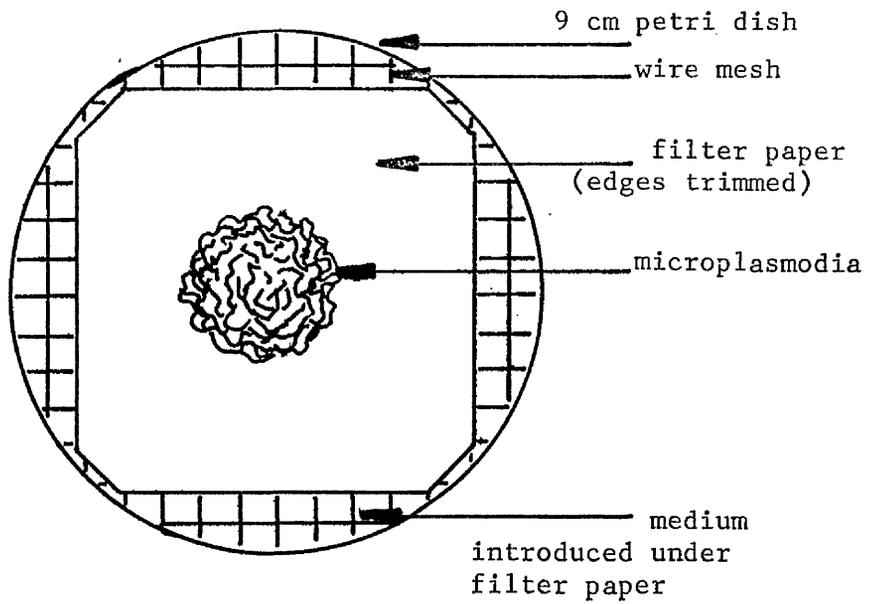
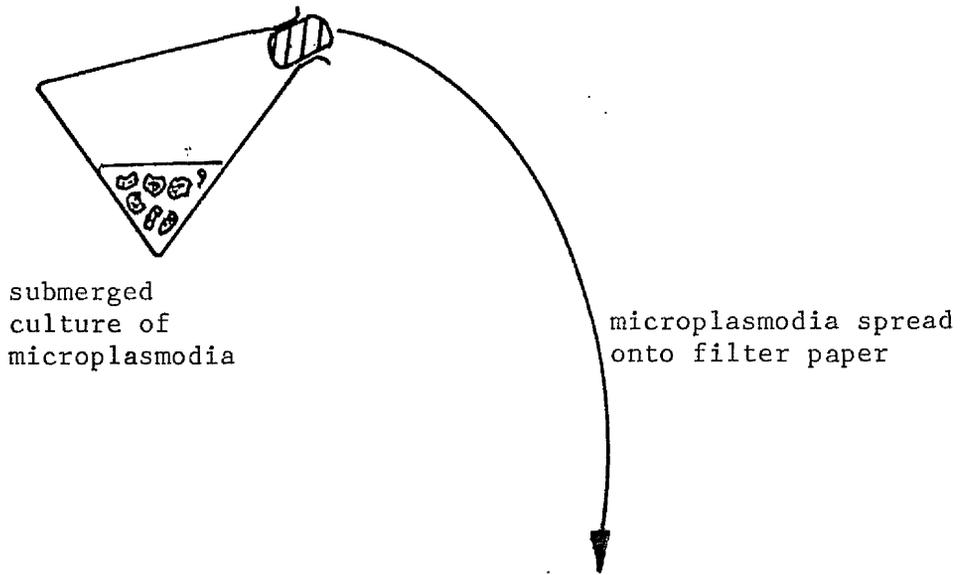
Table 3

Strain	genotype		
CL	<u>matAh</u> ,	<u>rac-1</u> ,	<u>fusA2</u>
LU216	<u>matA3</u> ,	<u>rac-2</u> ,	<u>fusA1</u>
LU276	<u>matAh</u> ,	<u>rac-1</u> ,	<u>fusA2</u>
	<u>npfA1</u> ,	<u>leu-1</u> ,	<u>whi-1</u>
LU862	<u>matA3</u> ,	<u>rac-2</u> ,	<u>fusA1</u>
	<u>eme</u> ^R		

Figure 3

Growth and sporulation of *P. polycephalum* on filter paper

Harvested microplasmodia of *P. polycephalum* were inoculated onto the surface of a filter paper supported by a wire mesh in a 9 cm petri dish. Growth or starvation medium was then pipetted under the filter paper.



use. Approximately 20 ml of SDM was introduced under the filter paper until the paper was soaked but the surface was not flooded. Filter papers were trimmed prior to sterilisation in order that the medium could be introduced beneath them more easily (Fig 3). Cultures were incubated in the dark at 26°C. Alternatively, harvested microplasmodia were introduced onto the surface of SDM agar (Appendix 1) on which they also formed a plasmodium when incubated at 26°C in the dark.

d) Transfer of surface plasmodia to liquid medium

On occasion it was necessary to subculture Physarum from a surface culture to liquid medium. This was achieved by removing some plasmodial growth with a sterile wire loop, from the surface of the filter paper or agar, and suspending it in 20 ml of SDM. The suspension was then incubated as a liquid culture.

e) Sporulation of *P. polycephalum*

Microplasmodia were prepared and spread on filter paper as described in section c) above, but sporulation medium (SM, Appendix 1) was introduced under the filter paper instead of SDM. After a period of incubation at 26°C in the dark sporulation was induced by exposing the starved plasmodium to light. If necessary plates were kept in the dark by wrapping them in aluminium foil. Surface cultures were incubated and illuminated in a Gallenkamp cooled incubator fitted with a timing device which allowed illumination for fixed periods at pre-determined times. Illumination was from 8W cool, white fluorescent lights. Petri dishes were placed between 10 and 20 cm from the lights. The intensity of the lights, at this distance, was measured with a 40x Optometre (United detector Technology Inc.) used with a radiometric filter and was found to be $250 \mu\text{Wcm}^{-2}$. During illumination the

temperature in the incubator was automatically lowered to 21^o to compensate for the local heating or "greenhouse effect" of the lights. Petri dishes for exposure to light of different wavelengths were illuminated using a Bausch and Lomb monochromator. The intensity of the light, at different wavelengths, was also measured.

Cultures could also be induced to sporulate by growing them on agar until nutrients became exhausted, sporulation could then be induced by illumination. Either SDM agar or dilute semi-defined medium agar (DSDM agar, Appendix 1) could be used. Nutrient became exhausted on DSDM agar more rapidly and consequently sporulation occurred earlier.

f) Harvesting sporangia and obtaining amoebae

After sporulation sporangia were left for 10 to 14 days to allow the spores to mature. After this time sporangia were harvested, dried in a plastic petri dish and then stored in screw cap bottles. To make a spore suspension a clump of sporangia were placed in a test tube containing 0.1 ml sterile distilled water. These were crushed with a sterile glass rod to release the spores which germinated when left to stand for 30 min in the water. A further 1.0 ml of water was then added and the germinated amoebae could then be cultured by spreading a drop of amoebal suspension together with a drop of Escherichia coli K12 suspension (see below) on a plate of liver infusion agar (LIA) (Appendix 1). Amoebal plaques were apparent in the lawn of bacterial growth after 4 to 5 days at 26^oC. These plates of amoebae could be stored at 4^oC as they encyst at this temperature and can survive for many months in this condition (Le Sturgeon, 1977).

g) Preparation of bacterial suspension

E. coli K12 was inoculated with a sterile loop onto nutrient agar (Oxoid) and incubated at 37^oC. After 48 h 5 ml of sterile distilled

water was added to the plate and the bacterial growth suspended in it by mixing with a sterile spreader. The bacterial suspension was then transferred to a test tube and stored at 4°C.

h) Storage of strains as amoebae

As mentioned in section f) above, plates of amoebae remain viable for several months when stored at 4°C. In some instances the strains of Physarum being used were maintained as amoebal stocks stored at 4°C. These stock cultures were subcultured every six months by transferring some amoebal growth with a sterile loop from the stock plate to a drop of E. coli K12 suspension on a fresh LIA plate. After spreading the suspension the plates were incubated at 26°C for 4 to 5 days and then stored at 4°C.

i) Freeze drying amoebae

As an alternative to storing amoebae on LIA at 4°C amoebae were freeze dried. Five plates of LIA were spread with a drop of amoebal suspension and a drop of bacterial suspension and incubated at 26°C for 4 to 5 days. One ml of sterile distilled water was then added to each plate and the amoebal growth suspended in it using a sterile spreader. The amoebal suspension was centrifuged at 1000 x g for 10 min at room temperature and the amoebae resuspended in 1.5 ml glucose horse serum (5 ml horse serum, 1.5 ml 30% (w/v) glucose), transferred to a Hemmings filter and centrifuged in a MSE super minor centrifuge at 1000 x g, 15 min. Two drops of the suspension were then added to each ampoule and these were restricted and dried on a 'speedivac' centrifugal freeze dryer (Edwards High Vacuum Ltd., Crawley, Sussex).

j) Obtaining plasmodia from amoebal plaques

As the Colonia strain of Physarum is apogamic, plasmodia

formed directly in each amoebal plaque. Plasmodia were cut from the agar with a sterile scalpel as they arose from amoebal plaques on LIA and the small agar blocks transferred to DSDM agar on which the plasmodia were allowed to migrate. A second transfer to another DSDM agar plate was often required to dissociate the plasmodium from the bacteria present for growth of the amoebae. Ultimately a piece of plasmodium was transferred to SDM agar on which better growth was obtained. If the bacteria proved difficult to eliminate then $250 \mu\text{gml}^{-1}$ streptomycin sulphate (Sigma) was incorporated into the agar. A plasmodium could be maintained by routine transfer of a piece of plasmodium to a fresh agar plate. Since cultures die within a few months if maintained this way they were eventually transferred to liquid culture for continued subculture (see section d).

k) Obtaining spherules from microplasmodia

Spherules were found to form in exhausted liquid growth cultures and these were used as an alternative to amoebae to maintain stock cultures. Ten day old liquid SDM cultures containing spherules were harvested by centrifugation ($1000 \times g$, 5 min) in a MSE super minor centrifuge. The spherules were washed twice in sterile distilled water and then spread onto Whatman 50 filter paper and allowed to dry. When completely dry the filter paper was aseptically cut into strips and the strips stored dessicated at 4°C in screw cap bottles.

l) Obtaining plasmodia from spherules

To regenerate a plasmodium from spherules, a filter paper strip supporting spherules was placed in a test tube containing 10 ml of SDM. The filter paper adhered to the side of the tube and was only semi-submerged in the medium. When incubated at 26°C in the dark plasmodia formed at the liquid interface and migrated on to the side of

the tube. A piece of plasmodia could then be transferred to liquid medium with a sterile loop.

2 Measurement of growth of microplasmodia in liquid medium

Growth was assayed by measuring the amount of yellow pigment present in the microplasmodia (Daniel & Baldwin, 1964; Dee & Poulter, 1970). Pigment was extracted by adding 20 ml of 4% TCA/acetone (8 ml 100% (w/v) trichloroacetic acid, 92 ml acetone, 100 ml distilled water) to a 2 ml sample of microplasmodia, mixing on a whirlimixer (Fisons) and allowing it to stand for 10 min at room temperature. After centrifugation (1000 x g, 5 min) in a MSE centrifuge the absorbance of the supernatant was determined at 400 nm in a Unicam SP600 spectrophotometer using a 1 cm light path. The amount of pigment produced was shown to be proportional to the amount of protein present (Fig 4). Protein present in a 2 ml sample of microplasmodia washed twice in sterile distilled water was determined by the Biuret method. 1 ml of 3N NaOH was added to the sample and it was boiled for 5 min, 1 ml of 2.5% (w/v) $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ was then added to the cooled sample and it was left to stand for 5 min. After centrifugation the absorbance of the supernatant at 555 nm was determined. Bovine serum albumin (Sigma) was used at concentrations of 2 mgml^{-1} to 8 mgml^{-1} to construct a standard curve.

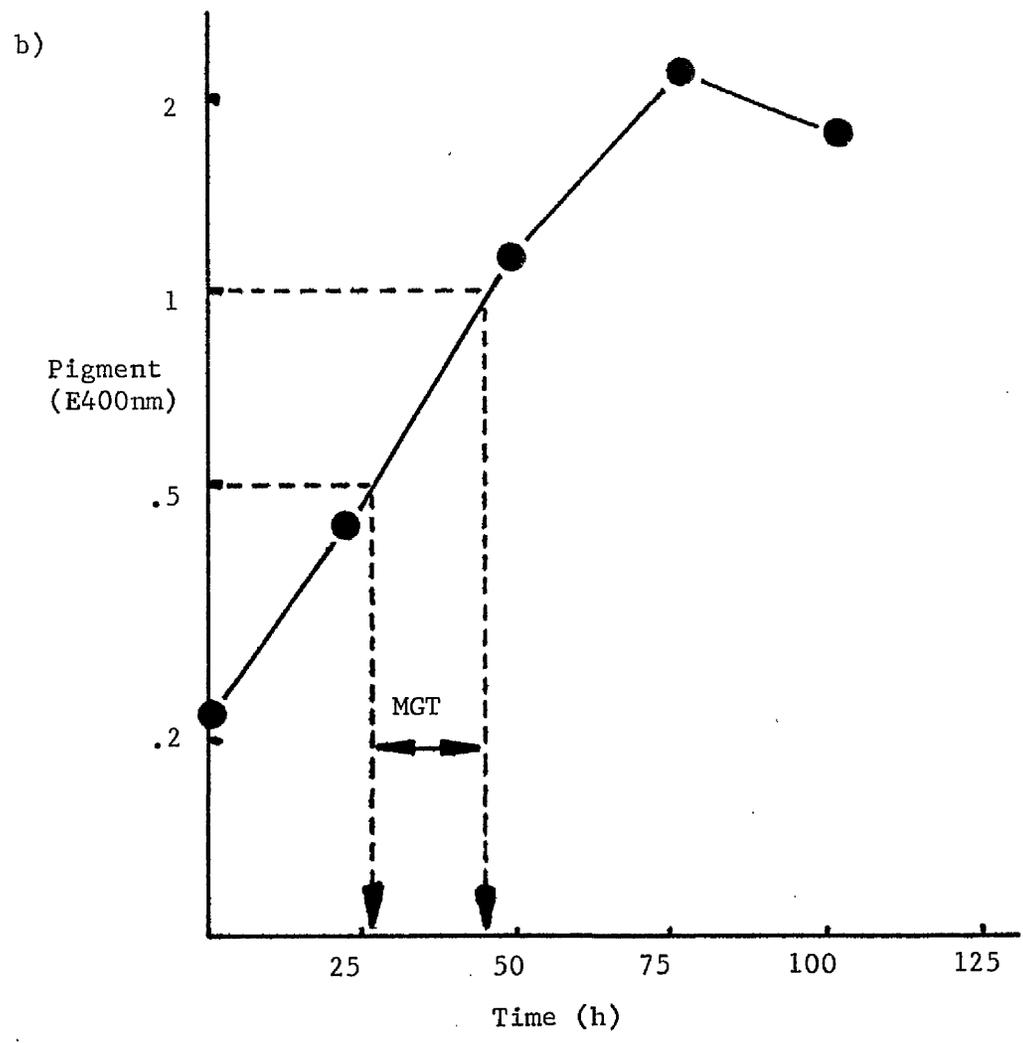
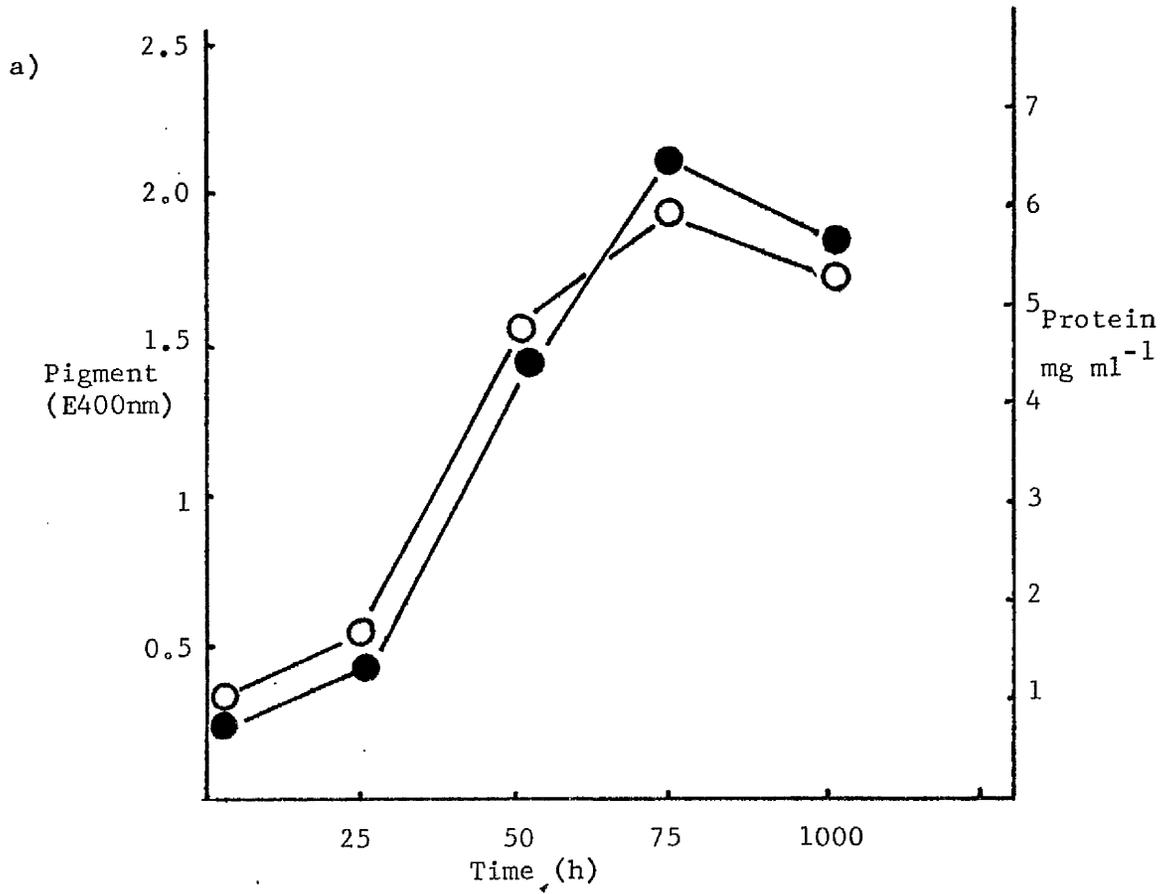
3 Microscopic observation of nuclei

A small piece (approx 0.5 cm^2) of the plasmodium from which nuclei were to be observed was transferred onto a glass slide. This was spread across the slide by scraping the edge of a second slide across its surface. The plasmodium was fixed by placing in absolute ethanol for 15 sec. A drop of 50:50 glycerol/alcohol was then added

Figure 4

Measurement of growth of microplasmodia in liquid culture

- a) Growth of microplasmodia of P. polycephalum in shaken liquid culture was measured by assay of protein by the Biuret reaction (○—○) and by extraction of pigment (●—●)
- b) Growth of microplasmodia measured by extraction of pigment plotted on log scale to demonstrate exponential growth.
- MGT = mean generation time calculated as the time taken to double the amount of extracted pigment when the plasmodium was growing exponentially, in this case calculated to be 20 h.



to the preparation and a coverslip placed on top. Nuclei were observed at x 1000 magnification on a light microscope (Vickers Instruments).

4 Mutagenesis of amoebae and selection of mutants

a) Mutagenesis of amoebae

Amoebae from a stock culture were plated onto LIA with a sterile loop (see section 1h). After 4 to 5 days incubation at 26°C the amoebal growth on the plate was harvested by adding 2 ml of sterile distilled water to the plate and suspending the growth in the water with a sterile spreader. The amoebal suspension was then transferred to a tube. With sterile Pasteur pipettes one drop of amoebal suspension and one drop of bacterial suspension were transferred to each of two plates of LIA, and after spreading the amoebae and bacteria the plates were incubated at 26°C for 4 to 5 days. The growth on these plates was then harvested as above and the amoebal suspensions transferred to two tubes. These suspensions were centrifuged (1000 x g, 5 min) at room temperature in a MSE super minor centrifuge. One amoebal pellet was resuspended in 5 ml 0.05M KH_2PO_4 (pH 7.0) containing 250 μgml^{-1} N-methyl N'-nitro N-nitroso guanidine (N.T.G., Aldrich Chemical Co. Ltd., Dorset, England); control amoebae were resuspended in KH_2PO_4 alone. These were incubated at 26°C for 30 min. The amoebae were then centrifuged (1000 x g, 5 min) in a MSE super minor centrifuge and resuspended in 5 ml of thiosulphate buffer (2% (w/v) $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ + 0.1% (w/v) Na_2CO_3) in which they were incubated for 10 min at 26°C. The amoebae were centrifuged again (1000 x g, 5 min) and resuspended in liver infusion broth (LIB, Appendix 1) and incubated at 26°C for 10 min. The amoebae were centrifuged and incubated for 10 min in LIB again. The amoebae were finally centrifuged and resuspended in LIB and incubated at 26°C for a post-mutational growth period of 48 h

which allowed expression of mutations before the amoebae were plated under selective conditions.

b) Killing curve for amoebae in NTG

In order to determine what percentage of the amoebae were killed after a given exposure to NTG a killing curve was constructed. Amoebae were suspended in a known volume of KH_2PO_4 + NTG, at 10 min intervals samples were removed (0.05-0.15 ml) and spread on LIA along with a drop of bacterial suspension using a sterile spreader. The number of amoebae present was calculated by counting the number of plaques produced and multiplying to the original volume.

c) Selection for drug resistance

After the 48 h growth period in LIB one drop of the amoebal suspension was spread with one drop of bacterial suspension on LIA containing the required drug concentration. Cycloheximide (Sigma) was used at $4 \mu\text{gml}^{-1}$ and emetine (Sigma) at $30 \mu\text{gml}^{-1}$. The drugs were prepared as concentrated solutions in sterile distilled water and then added at the required concentration to the molten agar immediately before plates were poured.

d) Selection for temperature sensitivity

After the 48 h growth period a range of volumes (0.05 ml to 0.25 ml) of the amoebal suspension were spread on LIA in order to obtain single plaques. Amoebae were then cloned by selecting single isolated plaques with the aid of a plate microscope (Vickers Instr., magnification x2). With a sterile loop some growth was removed from the selected plaque and transferred to a drop of bacterial suspension on a LIA plate. This was then spread with a sterile spreader and incubated at 26°C . When plaques were visible on this plate amoebae were re-cloned by selecting

a single plaque and replating as above and incubating at 26°C. Finally one plaque was selected from this plate and using a sterile loop with the aid of the plate microscope some growth was transferred to each of two plates of LIA and spread in a drop of bacterial suspension. One of these plates was incubated at the normal incubation temperature, 26°C, and the other at 31°C which was the highest temperature at which wild type amoebae would grow. Amoebae showing growth only at 26°C were selected as temperature sensitive mutants. To test plasmodia from these amoebae for temperature sensitivity plaques growing only at 26°C were allowed to form plasmodia on DSDM agar and then established on SDM agar (see section 1j). Once established on SDM agar a piece of plasmodium was placed on each of two SDM agar plates, one incubated at 26°C and the other at 31°C. Growth of plasmodia at these temperatures was observed visually for two to three days.

e) Selection for sporulation defective strains

i) Multi-well plates

2.5 ml DSDM agar was added to each of the wells of a sterile multi-well plate (Flow Labs, Irvine). These plates contained 24 wells each of 3.5 ml capacity and were fitted with a lid to prevent drying out of the agar. After mutagenesis cloned amoebae were selected and spread by means of a sterile loop in each well with a drop of bacterial suspension. These were incubated at 26°C and plasmodia formed after 6 to 7 days. Between 8 and 10 days after inoculation the plates were illuminated for 1 hour in the Gallenkamp cooled incubator and whether plasmodia sporulated or not was noted.

ii) Individual petri dishes

In addition to being grown in wells of agar, cloned amoebae were also grown on individual 9 cm or 5 cm plates of DSDM agar and

allowed to form plasmodia. The smaller plates were most frequently used when screening large numbers of amoebae. Once an established plasmodium on SDM was obtained (see section 1j) it was used to inoculate several plates of DSDM agar which were incubated at 26°C. Between 6 and 8 days after inoculation starved plasmodia were illuminated for 1 hour in the Gallenkamp cooled incubator and the number which sporulated recorded. Those which did not sporulate or showed abnormal sporangia formation were selected as being potentially sporulation defective. Sporulation defective strains obtained in this way had to be maintained as amoebae (section 1h), whilst the plasmodia which they formed were tested for their sporulation capacity. This was to ensure that amoebae of strains subsequently found to be sporulation defective were available for genetic analysis, otherwise amoebae of these strains would not have been available and the strains would have been lost. These strains could have been stored as spherules but once again amoebae would not then have been available.

iii) Continuous subculture

Strains which were sporulation defective were obtained from cultures of microplasmodia which had been subcultured for periods of six months or longer. When it became apparent that cultures were becoming sporulation deficient they were no longer used for sporulation experiments, but were retained as sporulation defective strains. These cultures were either maintained as microplasmodial cultures or as spherules derived from these cultures.

5 Genetic analysis

a) Crossing amoebae

Each of the amoebal strains to be crossed was subcultured from stock amoebal cultures onto LIA and incubated for 4 days at 26°C.

These actively growing cultures were used to carry out the cross. Bacterial suspension (0.2 ml) was added to the centre of a DSDM agar plate and using a sterile loop the first and then the second amoebal strain was mixed into the E. coli K12 as a puddle in the centre of the plate. After incubation at 26°C for 4 to 5 days plasmodia were observed migrating from the amoebal plaques. These were selected and established on SDM agar (section 1j). When carrying out a cross which involved the matAh allele plates were incubated at 31°C in order to minimise the number of plasmodia arising spontaneously, as selfed plasmodia, and not from crossed amoebae (Honey et al, 1979).

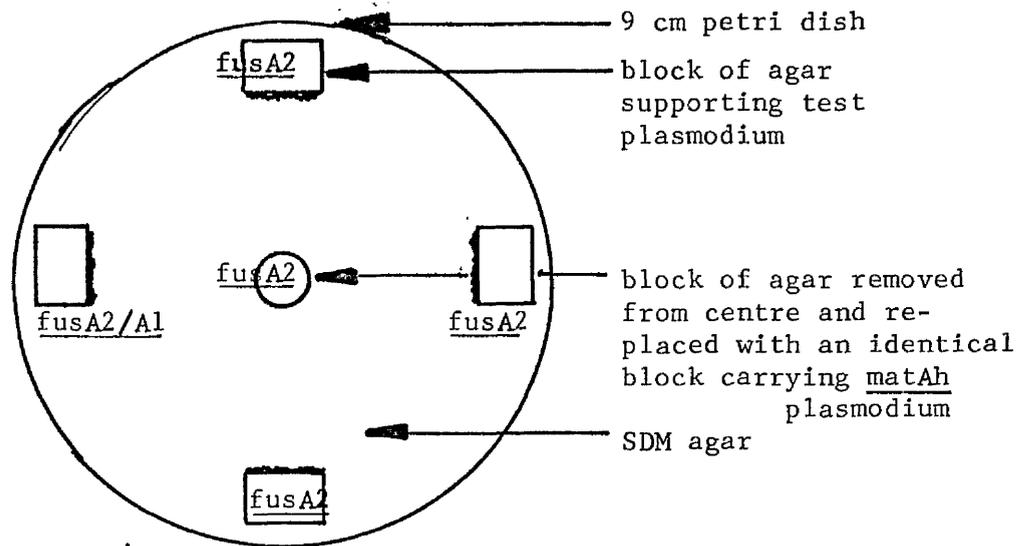
b) Plasmodial fusion test

Only plasmodia of the same fusion type are able to fuse, plasmodia with different fusion alleles remain separated when they come in contact with one another. This fact was used to distinguish plasmodia which arose spontaneously from those which had resulted from the amoebal cross. Two fusion alleles were involved, fusA2 and fusA1; matAh strains carry the fusA2 marker and consequently plasmodia which arise spontaneously are fusA2. One of the strains used in a cross carried the fusA1 marker and consequently plasmodia arising from crossed amoebae were fusA1/fusA2. These plasmodia were unable to fuse with the selfed fusA2 plasmodia. Hence crossed plasmodia could be distinguished from selfed plasmodia. Plasmodia selected from the cross (see section 4a) and established on SDM were used to carry out fusion tests to distinguish the crossed plasmodia. Four plasmodia were tested on one agar plate (Fig 5) by transferring blocks of agar supporting the test plasmodia to a SDM agar plate and arranging them around the edge of the plate. A block of agar supporting the apogamic fusA2 plasmodia, CL which had been cultured separately, was placed in the centre (Fig 5). After incubation at 26°C

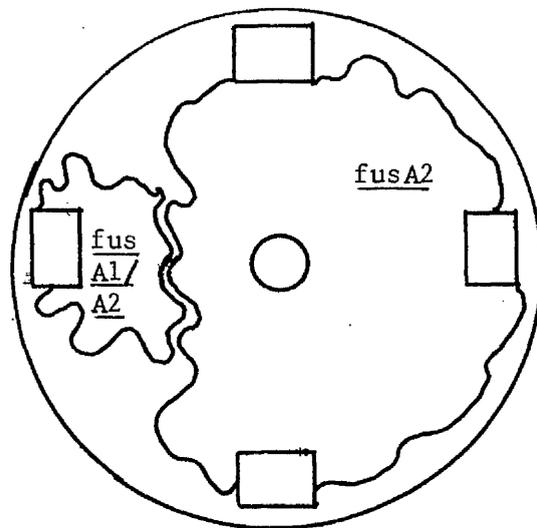
Figure 5

Plasmodial fusion test

Selfed fusA2 plasmodia were distinguished from crossed fusA2/fusA1 plasmodia since only selfed plasmodia were able to fuse with CL plasmodia which are fusA2



24 h at 26°C



for 24 h plasmodia were examined at regular intervals over a period of several hours using a plate microscope (Vickers, magnification x2). Fusion was only considered positive if veins could be seen running from one plasmodium to another and clear protoplasmic streaming could be seen between the two. If no fusion with CL occurred plasmodia were selected as heterozygous crossed strains and were transferred to fresh SDM agar.

c) Analysis of progeny from a cross

Once plasmodia were selected as crossed and not selfed plasmodia they were allowed to sporulate on DSDM agar. The mature sporangia were harvested and amoebae obtained from the spores (section 1e). The amoebae were then cloned by selecting single plaques (section 4d). The amoebae obtained and the plasmodia arising from those carrying the matAh allele (section 1j) were then tested for the markers involved in the cross. Table 4 illustrates the markers used and how they were distinguished in the cloned amoebae and the plasmodia they gave rise to.

6 Viability of spores

Spores obtained from diploid plasmodia germinate with greater efficiency than those obtained from haploid CL plasmodia (Laffler & Dove, 1977). This observation was used to check the strains used. A spore suspension was made in a known volume of water (section 1f). Using a haemocytometer (Neubauer, Hawksley) the number of spores present was calculated. A range of volumes (0.05 ml - 0.25 ml) of the suspension were then plated onto LIA + bacterial suspension and incubated at 26°C for 4 to 5 days. The number of plaques were then counted and the percentage of the original spores present which were viable was calculated.

7 Preparation of mitosis and DNA synthesis inhibitors

The inhibitors of mitosis colchicine and nocodazole were used.

Table 4

Markers and methods of screening

Marker	screening procedure
<u>matAh</u>	Only those amoebae carrying <u>matAh</u> could form plasmodia in clones (Cooke & Dee, 1975)
<u>npf</u>	Amoebae carrying <u>npf</u> were unable to form plasmodia in clones at 30°C but could form plasmodia at 22°C (Anderson & Dee, 1977)
<u>fus</u>	Only plasmodia carrying the same fusion genotype fused. Selected using the plasmodial fusion test (Fig 5) (Poulter & Dee, 1968)
<u>leu</u>	Plasmodia carrying <u>leu-1</u> were unable to grow on DM-L agar (Appendix 1) (Cooke & Dee, 1975)
<u>whi</u>	<u>whi-1</u> plasmodia are white (Anderson, 1977)
<u>spo</u>	Sporulation capacity of plasmodia on DSDM agar (see section 3d(ii)).

Colchicine (Sigma) was stored as a powder at 4°C and added directly to media immediately before use. A stock 5 mgml⁻¹ solution of nocodazole (methyl-5-(2thienylcarbonyl)-1H-benzimidazol-2YL-carbamate, Sigma) was prepared in DMSO (dimethyl sulphoxide) and stored frozen at -20°C. Three inhibitors of DNA synthesis were used, BUdR (5 Bromo-2'-deoxy uridine, Sigma), AraC (cytosine-1-β-D Arabinofuranoside, Sigma) and hydroxyurea (Sigma). All three were stored as powders, BUdR at -20°C, AraC and hydroxyurea at 4°C. These inhibitors were added directly to media to give the required final concentration immediately before use. In addition the protein synthesis inhibitor cycloheximide (Sigma) was used. This was stored as a powder at 4°C and prepared by adding directly to media immediately before use.

8 Measurement of DNA synthesis

Uptake of [methyl-³H] thymidine (47 Ci mmol⁻¹, Amersham) into growing and starving plasmodia was studied. Small pieces, approximately 1 cm square, of filter paper supporting the plasmodia were cut out with sterile scissors and transferred to a wire grid in a petri dish containing 20 ml SDM or SM with thymidine added at a concentration of 0.2 μCi ml⁻¹. These were incubated at 26°C for 30 min after which they were immediately plunged into 20 ml of cold 4% TCA acetone. This procedure removed all the pigment from the plasmodia on the filter paper. After centrifugation, 1000 x g, 5 min in a MSE centrifuge at room temperature, the absorbance of the supernatant was determined at 400 nm. This was used to estimate the amount of material collected in each case in order that the final radioactive count could be standardised. The precipitate, now removed from the filter paper, was washed twice in 0.25M perchloric acid containing 100 μgml⁻¹ thymidine (Sigma) and finally solubilised in 2 ml 0.4M NaOH. Toluene based scintillant (Triton X-100 1 litre, toluene

2 litres, 8 g 2,5 diphenyloxazole (Koch Light Labs Ltd), 0.2 g 1,4-Di-2-(4methyl-5phenyl oxazole) benzene (BDH) was then added to 1 ml of the sample and the radioactivity counted in a Packard TRI-CARB 300C scintillation counter.

RESULTS

1 Sporulation capacity during growth of microplasmodia

Microplasmodia harvested at various points during the growth cycle were tested for their ability to sporulate. Microplasmodia used for these experiments were grown in SDM and generally had a mean generation time of about 20 h (Fig 4b). It was shown that microplasmodia harvested at the end of exponential growth gave the best sporulation frequency (Table 5). It was noted that this requirement for harvesting microplasmodia at the end of exponential growth could be overcome to some extent by allowing renewed growth of the microplasmodia as a surface plasmodium for 24 h before introducing sporulation medium (SM) (Table 5). This was done by introducing growth medium under the filter for 24 h and then transferring the surface plasmodium on the filter to SM. It was concluded that as long as microplasmodia were harvested at the end of exponential growth there was no requirement for growth as a surface plasmodium to ensure successful sporulation. In addition, microplasmodia harvested at the end of exponential growth sporulated earlier than those harvested in stationary phase (Table 5). In general they sporulated after illumination at 72 h during starvation whilst the others sporulated after illumination at 96 h or later.

2 Effect of volume of microplasmodia harvested

Since 5 ml volumes of microplasmodia were used in all cases in the initial experiments, and samples taken early in the growth cycle would have contained fewer microplasmodia, it was possible that sporulation was connected with the number of microplasmodia being harvested. By varying the volume harvested according to the $E_{400\text{nm}}$ of the sample the mass of microplasmodia harvested at intervals during the growth cycle was standardised (Table 6). This experiment was also conducted

Table 5

Sporulation frequency of a standard volume of microplasmodia harvested at different points during the growth cycle

Four x 5 ml samples of microplasmodia were harvested at various times during the growth cycle and spread on filter paper (see Methods, section 1e). They were then either directly exposed to SM or were allowed to grow on SDM for 24 h before transfer to SM. After illumination for 30 min at 72 h during starvation the number sporulating out of each set of four was recorded. Those which failed to sporulate were illuminated for a further 30 min at 96 h and the final sporulation frequency recorded. In all cases successful sporulation was only considered to have taken place if fully mature sporangia developed within 24 h of illumination.

Age of culture (h)	E_{400nm}	Volume of culture harvested (ml)	Sporulation frequency	
			No growth on filter	24 h growth on filter
24	0.17	5	0/4	1/4
54	0.72	5	0/4	3/4
76	1.80	5	4/4 (4/4)	4/4
97	1.72	5	3/4 (0/4)	3/4

Figures in brackets indicate the number which sporulated after illumination at 72 h.

Table 6

Sporulation frequency of a standard mass of microplasmodia harvested at different points during the growth cycle

Experimental procedure as for Table 1 except the volume of microplasmodia harvested at each point in the growth curve was varied according to the $E_{400\text{nm}}$ reading of extracted pigment such that a standard mass of microplasmodia was harvested at each point.

Age of culture (h)	$E_{400\text{nm}}$	Volume of culture harvested (ml)	Sporulation frequency	
			No growth on filter	24 h growth on filter
24	0.27	14.0	0/4	2/4
48	0.69	5.5	0/4	4/4
96	1.80	2.1	4/4 (4/4)	3/4
124	1.54	2.4	2/4 (0/4)	4/4

Figures in brackets indicate the number which sporulated after illumination at 72 h.

with and without an overnight growth step on the filter. Since the results obtained were similar to those in Table 5 it was concluded that alteration of the mass of microplasmodia harvested was not responsible for the variation in sporulation capacity. This was confirmed by allowing plasmodia to form from different volumes of microplasmodia harvested at the end of exponential growth when sporulation capacity was at its peak. Sporulation occurred regardless of the volume harvested, but the number of fruiting bodies formed per filter increased with increasing volume of microplasmodia (Table 7). Similarly, reducing the volume of sporulation medium used, by using a smaller petri dish, had no effect on sporulation frequency (Table 7). The ratio of sporulation medium to microplasmodial mass, therefore, had no effect on sporulation frequency. Successive transfers of plasmodia to fresh SM at intervals during starvation also had no effect on sporulation frequency (Table 8). These experiments were conducted to check the theory of Wilkins & Reynolds (1979) since either altering the ratio of plasmodial mass to sporulation medium or changing the sporulation medium should improve sporulation frequencies according to these authors (see introduction). It had been established therefore that the microplasmodia should be harvested at the end of exponential growth and at this point an overnight growth step was not required nor was the volume harvested of any consequence. A volume of 5 ml was used routinely in all subsequent experiments.

3 Length of starvation period in the dark

Nutrient deprivation and illumination were known to be required for sporulation of P. polycephalum M₃C strain, but the minimum length of each requirement was uncertain for the CL strain. In order that the length of starvation could be determined the exact time of

Table 7

Effect on sporulation of varying the volume of microplasmodia harvested at the end of exponential growth

The volume of microplasmodia harvested at the end of exponential growth was varied. Four samples of each volume were harvested. After illumination at 72 h the total number sporulating and the number of fruiting bodies produced per plasmodium were recorded. Alternatively, the volume of SM used was reduced by using a smaller, 5 cm, petri dish and the effect on sporulation frequency of this change in the ratio of microplasmodial mass to SM volume noted.

Volume harvested (ml)	SM (ml)	Sporulation frequency	Average no. of fruiting bodies produced per plasmodium
0.5	20	4/4	17
1.0	20	4/4	40
2.5	20	4/4	60
5.0	20	4/4	121
5.0	2	4/4	N.D.

N.D. not determined

Table 8

Effect on sporulation of transfer of plasmodia to fresh sporulation
medium at intervals during starvation

Starving plasmodia were transferred to a grid underlaid with SM as a washing step and then transferred to another grid and underlaid with fresh SM at the times indicated. Plasmodia were illuminated for 30 min at 72 h during starvation and the number which sporulated within 24 h recorded.

Time (h) of transfer to fresh SM	Sporulation frequency
24	5/5
48	5/5
72	5/5
74	5/5
24, 48 & 72*	5/5
medium unchanged	5/5

* these cultures were transferred to fresh SM
sequentially at 24 h, 48 h and 72 h.

initiation of starvation had to be established. Previous workers (Daniel & Rusch, 1962a,b; Daniel & Baldwin, 1964; Daniel, 1966; Sauer et al, 1969a) had always included a period of several hours in the absence of media to allow microplasmodia to fuse and form a plasmodium. In order that the time of initiation of starvation could be related more closely to the addition of sporulation medium it was desirable to reduce this period as far as possible. Accordingly, after microplasmodia were spread on the filter paper various times ranging from 0 to 180 min in the absence of medium were allowed for microplasmodia to fuse before addition of SM. Sporulation was found to occur just as readily when essentially no fusion time was allowed, other than the short time between spreading microplasmodia and addition of SM beneath the grid. Consequently no fusion period was included in subsequent experiments and starvation was initiated as soon as microplasmodia were layered onto the filter paper and SM was introduced beneath the filter.

Using this method it was established that a period of 72 h starvation in the dark was required before a single light pulse would initiate morphogenesis in all cultures (Table 9). Shorter periods of starvation never allowed all of the cultures to sporulate, although from 60 h onward illumination produced sporulation in an increasing fraction of the plasmodia tested. It can also be seen that extended starvation, 120 h or longer, resulted in a situation where cultures were no longer capable of being induced to sporulate by illumination. In fact the morphology of these cultures also changed; they became a darker yellow and showed less distinct veins and appeared to have died. The minimum period of 72 h starvation was confirmed in a separate experiment where 40 plasmodia were starved for 72 h in the dark and then illuminated; all sporulated within 24 h of illumination.

Table 9

Sporulation frequency after various starvation times followed by illumination

Forty four plasmodia were prepared in the normal manner and incubated for various lengths of time in the dark before being illuminated for 30 min. Cultures were wrapped in aluminium foil to ensure they were kept dark and at intervals 4 plasmodia were removed and illuminated. The number completing sporulation within 24 h of illumination was recorded.

Length of starvation in the dark (h)	Sporulation frequency
55	0/4
60	1/4
65	2/4
69	2/4
70	3/4
72	4/4
74	4/4
96	4/4
120	0/4
144	0/4
192	0/4

Whilst investigating the starvation period it was noted that if starving plasmodia were given light pulses of ^{30 min}~~1 h~~ duration at intervals during starvation, sporulation of some plasmodia would invariably occur earlier than others (Fig 6). Plasmodia were frequently observed to sporulate after only 40 to 50 h starvation whilst on one occasion some plasmodia sporulated after only 36 h starvation. There was no way of telling in advance which of a series of plasmodia would sporulate early. It was estimated that under these conditions of intermittent illumination approximately 50% of plasmodia would sporulate early, whilst the remainder would sporulate after illumination at 72 h. Consequently it was decided that a period of 72 h starvation in the dark would be used since illumination after this period reliably induced all cultures to sporulate.

4 Length of time of illumination

Having decided to use a period of 72 h starvation it was necessary to determine what length of time of illumination was then required to induce sporulation. Illumination was shown to be an absolute requirement since cultures kept continuously in the dark did not sporulate (Table 10). Once again of four plasmodia given several light pulses during starvation two sporulated early whilst one of the four left continuously in daylight sporulated early. All four cultures illuminated after 72 h in the dark sporulated. In all these instances a 30 min light pulse was used and it had next to be determined what minimum length of time of illumination was required. Plasmodia were starved for 72 h in the dark and then illuminated for between 5 and 60 min (Table 11). A light pulse of as little as 5 min was sufficient to produce sporulation, but to ensure sufficient illumination in all experiments it was decided to routinely use a period of 30 min illumination.

Figure 6

Effect on sporulation of periods of illumination given at intervals during starvation

Ten plasmodia were prepared for each experiment.

All of the plasmodia were illuminated for 30 min at intervals during starvation and the number sporulating within 24 h of a given light pulse was recorded.

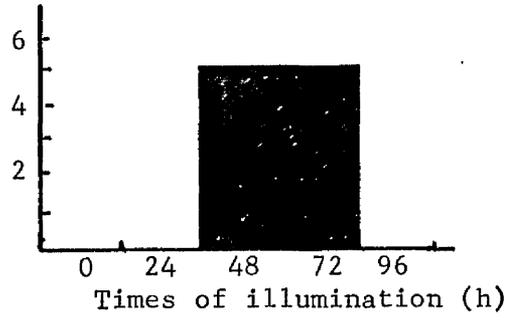
Illumination was continued at 24 h intervals until all 10 cultures in the experiment had sporulated.

Times of illumination (h): Times during starvation when all cultures in an experiment were illuminated.

Sporulation: number of cultures out of 10 which had sporulated within 24 h of a given light pulse.

a)

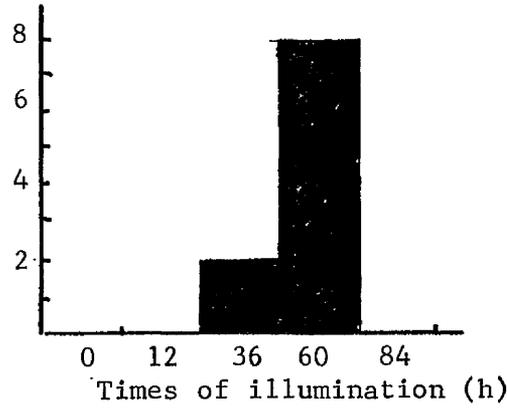
Number
sporulating



Experiment 1

b)

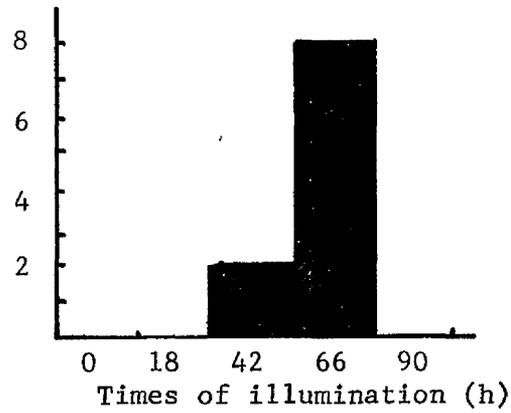
Number
sporulating



Experiment 2

c)

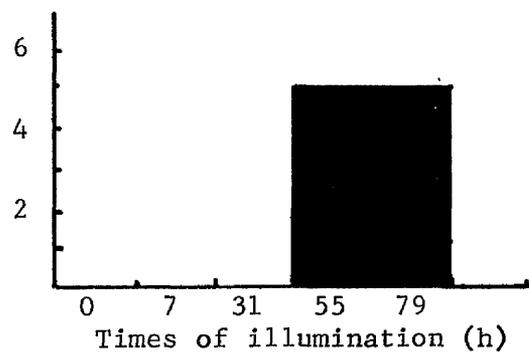
Number
sporulating



Experiment 3

d)

Number
sporulating



Experiment 4

Table 10

Effect of various illumination conditions on sporulation

Plasmodia were prepared for sporulation and were then either wrapped in aluminium foil or left on the bench at room temperature to be exposed to daylight continuously (these received no additional illumination). Of the plasmodia wrapped in foil four were left for 72 h in the dark and then illuminated for 30 min, four were exposed to 30 min illumination every 24 h and four were never illuminated, being kept wrapped in foil for longer than 124 h. These were checked daily after 72 h, under dark room safe light conditions, for sporulation.

illumination	Sporulation frequency
none	0/4
daylight	4/4 (1/4)
30 min pulse	
every 24 h	4/4 (2/4)
One 30 min	
pulse after	
72 h in the dark	4/4

Figures in brackets represent the number which sporulated before 72 h of starvation.

Table 11

Length of time of illumination required to produce sporulation
after 72 h starvation in the dark

After 72 h starvation in the dark groups of 5 plasmodia were exposed to varying lengths of illumination. The number of plasmodia sporulating within 24 h of illumination was recorded.

Length of illumination (min)	Sporulation frequency
0	0/5
5	5/5
15	5/5
30	5/5
60	5/5

An attempt was made to determine which wavelengths of light were required to promote sporulation. Plasmodia which had been starved for 72 h in the dark were exposed to light of known wavelength from a monochromator and whether they subsequently sporulated or not was recorded. Of the range of wavelengths tested only 400 nm promoted some plasmodia to sporulate; however no specific wavelength was able to promote all cultures to sporulate like the control illumination (Table 12).

The routine procedure finally adopted was as follows. Five ml of microplasmodia were harvested at the end of exponential growth. These were spread on filter paper under which SM was added immediately. At the end of 72 h starvation in the dark plasmodia were illuminated for 30 min and this routinely resulted in 100% sporulation.

5 Loss of ability to sporulate

It had been reported previously that cultures of P. polycephalum lose the ability to sporulate after extensive subculture (Daniel & Baldwin, 1964; Hosoda, 1980). This fact also affected the results obtained here and must be taken into account when working with P. polycephalum. When several subcultures of the same CL strain were tested for their ability to sporulate considerable differences between them were observed (Table 13). Furthermore, cultures showing poor sporulation frequencies also sporulated later. In a separate experiment six plasmodia were prepared from subcultures A, B and C (see Table 14). All six of A sporulated after the light pulse at 72 h whilst only one of B and none of C did so, however five of B and four of C managed to sporulate after a second light pulse given at 96 h (Table 14). Clearly of the four subcultures considered here only one gave the expected 100% sporulation after illumination at 72 h. The other three exhibited a reduced capacity to sporulate and the plasmodia had a requirement for a longer period of

Table 12

Effect of different wavelengths of illumination on sporulation frequency

After 72 h starvation in the dark plasmodia were exposed to 30 min illumination of a specified wavelength, whether sporulation was promoted or not was recorded. Control cultures were illuminated for 30 min in the normal manner with white light.

Wavelength of illumination	Intensity of light per cm ² (μ W)	Sporulation frequency
600	125	0/6
550	100	0/6
500	90	0/6
450	50	0/6
400	20	2/6
control	250	6/6

Table 13

Sporulation competence of subcultures of *P. polycephalum* CL

Subculture:

- A. Regenerated from 6 month old spherules prepared from the CL strain when sporulating well.
- B. Regenerated from 14 month old spherules prepared from a subculture which was beginning to lose sporulation competence after repeated subculture as microplasmodia over a period of several months.
- C. Regenerated from freeze dried amoebae which were 3 months old. A subculture which was sporulating well was used to obtain spores and subsequently amoebae, which were then freeze dried (see Materials and Methods). The amoebae were reconstituted in LIB and then allowed to form plasmodia on LIA, the plasmodia were then transferred to SDM and subcultured as microplasmodia.
- D. CL strain supplied as amoebae by Dr. J. Dee (University of Leicester, Dept. of Genetics). The amoebae were allowed to form plasmodia as with subculture C.

Table 13

Plasmodia of the various subcultures were prepared in the normal way and starved for 72 h before being illuminated for 30 min. Cultures were given a further 30 min period of illumination at 96 h and the number sporulating within 24 h of this was recorded as the final sporulation frequency.

In some cases the amount of niacin and CaCO_3 added to the SM was increased, the number sporulating in these cases was recorded as above.

Additions to SM (mg ml^{-1})		Sporulation frequency			
Niacin	CaCO_3	A	B	C	D
none	none	3/6	-	-	-
none	1.0	6/6	-	-	-
* 0.1	1.0	6/6	2/3	0/3	1/3
0.2	2.0	-	3/3	2/3	3/3
0.3	3.0	-	1/3	1/3	2/3

* normal additions to SM.

Table 14

Time of sporulation of subcultures of the CL strain

Three subcultures, A, B and C, as described in Table 13 were illuminated for 30 min after 72 h of starvation and a further 30 min after 96 h starvation. The number sporulating within 24 h of each light pulse was recorded.

Subculture	Sporulation after illumination at	
	72 h	96 h
A	6/6	-
B	1/6	5/6
C	0/6	4/6

starvation in the dark before illumination was able to induce any to sporulate. Before using a culture of P. polycephalum CL to conduct studies on sporulation it must first, therefore, be established that the culture being used sporulates reliably. Once a culture which sporulated well was obtained it was stored as spherules. As soon as a subculture became unreliable with respect to sporulation it was then possible to regenerate that ability by starting a fresh culture from spherules. It was noted that this variation in ability to sporulate could be compensated for, to some extent, by increasing the amount of niacin and CaCO₃ added to the SM. Culture A was capable of sporulating equally well in the absence of niacin and to some extent in the absence of both niacin and CaCO₃, whilst the sporulation frequencies of the other three subcultures increased to some extent when the concentrations of niacin and CaCO₃ increased (Table 13). It should be noted however that although double concentrations of niacin and CaCO₃ improved the sporulation frequency, triple concentrations decreased it again.

Finally, it was apparent that even a good sporulating subculture would give poor results if anything had interfered with growth of the microplasmodia during the 2 to 3 subcultures prior to harvesting eg. if the culture had been contaminated; if a subculture was made from a very old or a very young culture or if the microplasmodia had only recently been regenerated from spherules. In order to achieve good sporulation results, cultures had to have been growing exponentially for 8 to 12 generations prior to starvation. If it had been established that a culture was a good sporulating subculture and had been growing well then sporulation could be reliably produced.

6 Time of commitment to sporulation

Having established a method for reproducibly achieving

sporulation of all starved plasmodia of the CL strain, the point when starved cultures became irreversibly committed to sporulation was determined. This was done by refeeding starving plasmodia with fresh growth medium at various times during starvation and noting whether cultures resumed growth or sporulated normally. Cultures became committed to sporulate between 4 and 5 h after illumination (Table 15). Up until this time sporulation could be prevented and the plasmodia would resume growth. The time of commitment was related to the time of illumination and not the overall length of starvation since altering the time of illumination altered the time of commitment (Fig 7). Hence cultures illuminated at 70 h, two hours earlier than normal also became committed two hours earlier than plasmodia illuminated at 72 h. Similarly, those illuminated at 75 h, three hours later than normal showed a concomitant 3 h delay in the time at which they became committed to sporulate.

In order to determine whether any particular constituent of the growth medium was responsible for preventing sporulation, starving cultures were refed with SM supplemented with D-glucose, L-glutamine, L-glutamate and $(\text{NH}_4)_2\text{SO}_4$ and it was noted whether sporulation was prevented (Table 16). These results indicated that a nitrogen source alone eg. $(\text{NH}_4)_2\text{SO}_4$ could not prevent sporulation if added during starvation, and it seemed clear that a carbon source was required. Another experiment indicated that glucose alone could prevent sporulation at any time up to 4 h after illumination (Table 17) at which time plasmodia were committed to sporulate in a similar way to those refed with growth medium (Table 15), although if anything plasmodia became refractory to the effect of glucose about 1 h earlier.

Table 15

Effect on sporulation of transferring cultures to growth medium during starvation

Starving plasmodia were refed with fresh growth medium at various times during starvation and whether cultures resumed growth or sporulated normally after illumination at 72 h was recorded.

Time (h) during starvation of transfer to growth medium	Sporulation frequency
48	0/5
67	0/5
71	0/5
73	0/5
74	0/5
75	0/5
76	0/5
77	5/5
78	5/5
control	5/5

Figure 7

Effect of altering the time of illumination on time of
commitment to sporulation

Starving plasmodia were refed with growth medium at various times during starvation as in Table 15.

Three separate experiments were conducted, the time of illumination being the only difference between them.

Plasmodia were illuminated at 70 h (●), 72 h (○) and 75 h (■) and whether sporulation occurred within 24 h was recorded.

These results are presented as a figure as opposed to a table, as in table 15, to aid interpretation of the effect of changing the time of illumination.

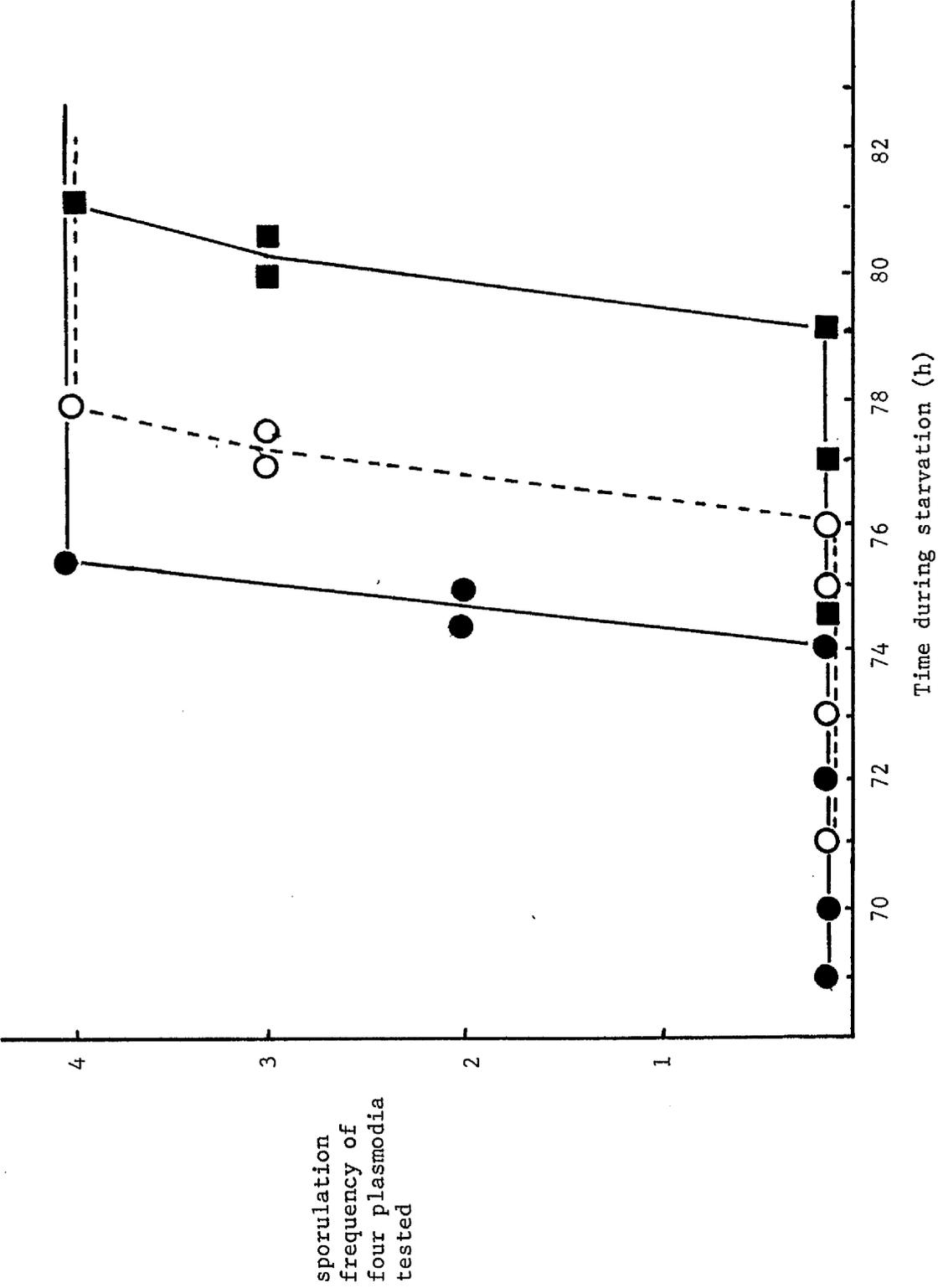


Table 16

Effect on sporulation of refeeding starving plasmodia with
D-glucose, L-glutamate, L-glutamine and $(\text{NH}_4)_2\text{SO}_4$

Starving plasmodia were refed at either 48 h or 73 h during starvation with one of the following; D-glucose (1% (w/v)), L-glutamine (0.35% (w/v)), L-glutamate (0.34% (w/v)) or $(\text{NH}_4)_2\text{SO}_4$ (0.26% (w/v)). After illumination at 72 h during starvation the sporulation frequency was recorded.

Concentrations used were equivalent to the concentration of each constituent present in growth medium.

Plasmodia refed with	Sporulation frequency after refeeding at	
	48 h	73 h
D-glucose	0/2	0/2
L-glutamate	0/2	0/2
L-glutamine	2/2 *	2/2 *
$(\text{NH}_4)_2\text{SO}_4$	2/2	2/2

* Delayed sporulation; sporangia were not completely developed within 24 h of illumination.

Table 17

Effect on sporulation of refeeding with D-glucose (1% (w/v))
during starvation

Cultures of starving plasmodia were refeed with D-glucose at various times during starvation. All plasmodia were illuminated at 72 h and whether sporulation occurred within 24 h noted.

<u>Time of addition of D-glucose during starvation (h)</u>	<u>Sporulation frequency</u>
48	0/4
70	0/4
74	0/4
75	1/4
76	4/4
77	4/4
control	4/4

7 Effect of inhibitors on growth and sporulation

As discussed in the introduction one aspect of sporulation which it was hoped could be investigated was the importance of DNA synthesis and mitosis. Inhibitors of these events were used to determine whether by blocking DNA synthesis or mitosis, sporulation could also be prevented. In conjunction with these inhibitor studies it was hoped to observe mitosis, if it occurred, microscopically. In preparations of growing plasmodia examined at x1000 magnification, nuclei could be distinguished as spherical objects but no intranuclear detail was apparent. Nuclei from plasmodia which had been grown on SDM containing the mitotic inhibitor nocodazole were also prepared and observed microscopically; these nuclei it was assumed would be arrested in mitosis. However, although these nuclei appeared different from normal nuclei, in that some details of internal structure were apparent, it was not clear how these could be distinguished as being in mitosis. Consequently it was never found possible to actually observe mitosis during starvation and so determine microscopically that mitosis did take place.

1) Inhibitors of mitosis

Initially colchicine was chosen as a mitotic inhibitor and the effect of this drug on growth of microplasmodia was tested. The inhibitor was added to the growth medium immediately before inoculation and growth assayed by measurement of pigment produced. Colchicine was found to have no effect on growth even at high concentrations (Fig 8). Consequently the effect of the benzimidazole derivative nocodazole was tested. Nocodazole was found to prevent growth of microplasmodia at a concentration of $20 \mu\text{gml}^{-1}$ (Fig 9). Nocodazole was prepared and stored as a 5mgml^{-1} solution in dimethyl sulphoxide (DMSO). The concentration of DMSO added to medium never exceeded 0.4% (equivalent concentration in

Figure 8

Effect of the mitotic inhibitor colchicine on growth
of microplasmodia

Microplasmodia were subcultured into SDM and SDM + 400 μgml^{-1} colchicine. Growth was measured by assaying extracted pigment from samples of microplasmodia.

- Normal medium (SDM)
- SDM + 400 μgml^{-1} colchicine.

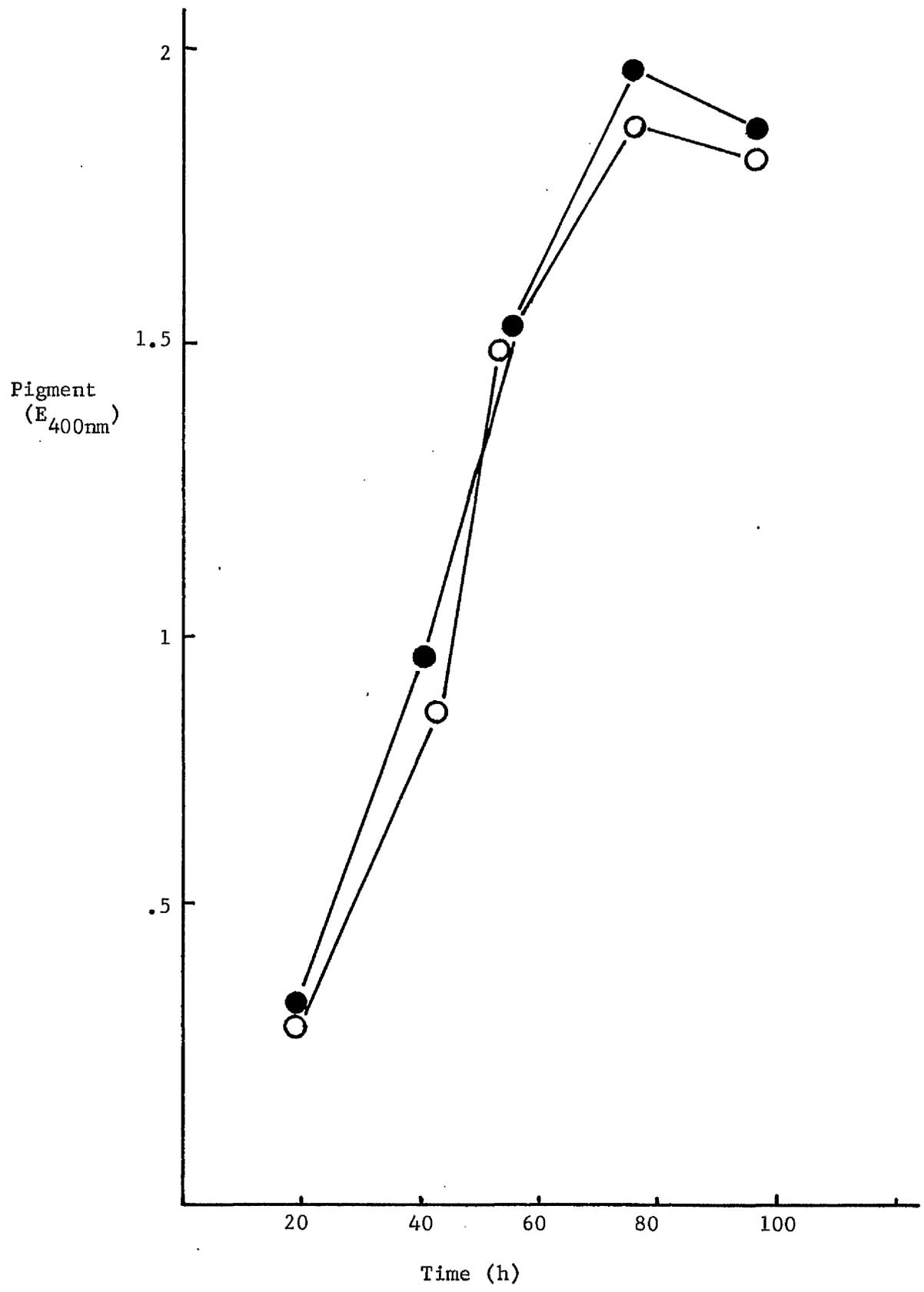
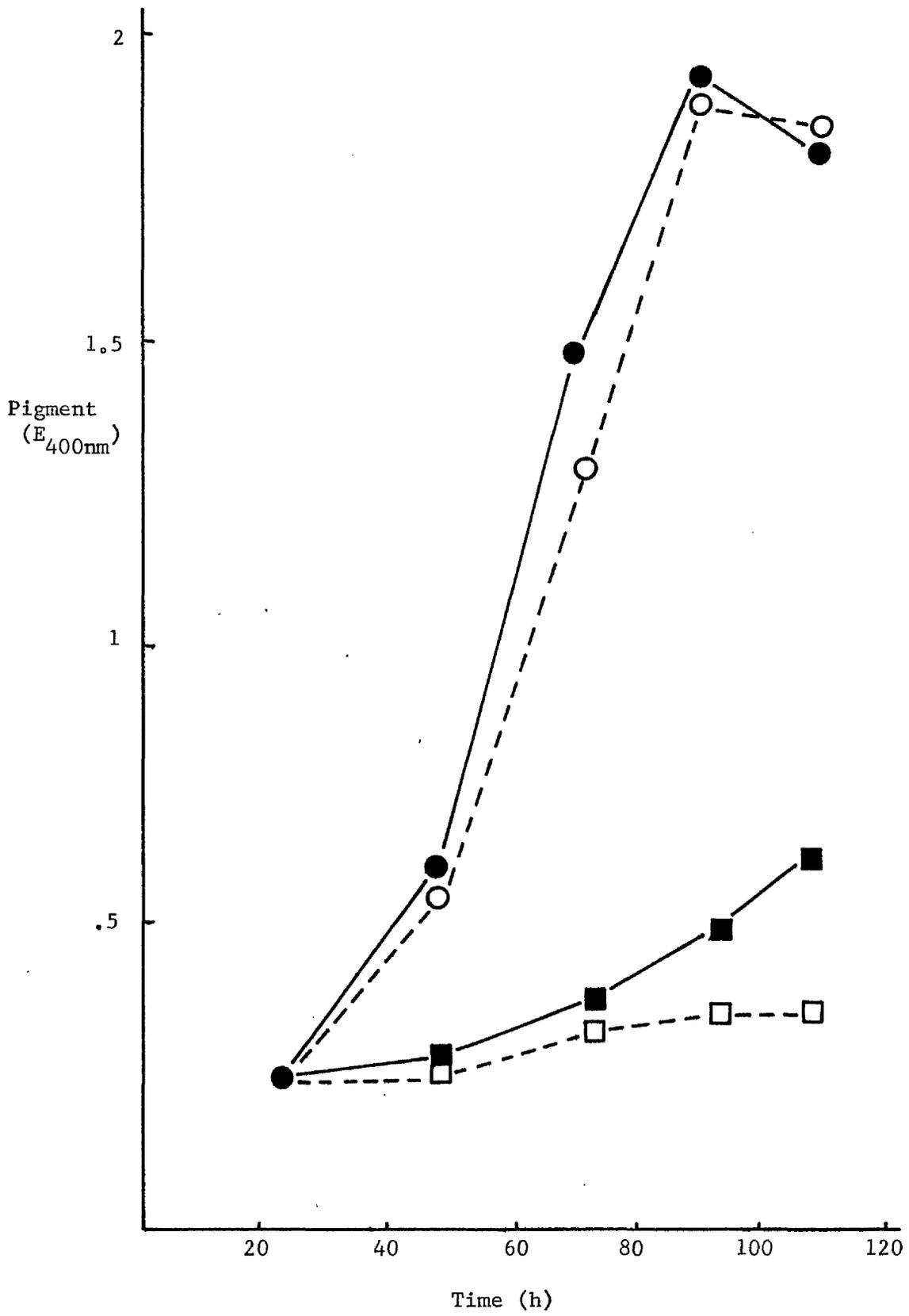


Figure 9

Effect of nocodazole on growth of microplasmodia

Growth of microplasmodia in SDM and SDM + nocodazole was measured by extracting the pigment from samples of microplasmodia. Since stock solutions of nocodazole were prepared in DMSO the effect of this on growth of microplasmodia was also tested.

- normal medium (SDM)
- SDM + DMSO (final concentration 0.4%)
- SDM + 1- μgml^{-1} nocodazole
- SDM + 20 μgml^{-1} nocodazole.



a 20 μgml^{-1} nocodazole solution) and this concentration of DMSO was shown to have no effect on growth of microplasmodia (Fig 9). Nocodazole at a concentration of 20 μgml^{-1} was chosen since the lower concentration of 10 μgml^{-1} resulted in some growth of microplasmodia after 72 h (Fig 9).

Nocodazole, therefore, was used as a mitotic inhibitor and its effect on sporulation was investigated. At intervals during starvation filter papers supporting the starving plasmodia were transferred to sporulation medium containing 20 μgml^{-1} nocodazole and the effect on sporulation noted. DMSO at the low concentration normally added with the nocodazole had no effect on sporulation. Nocodazole added prior to 48 h during starvation prevented sporulation but plasmodia began to escape the inhibition of sporulation after this time (Table 18). This observation might suggest that a mitosis necessary for sporulation occurred between 48 and 72 h during starvation. Nocodazole added at 65 h prevented the majority of cultures from sporulating which suggested that the mitosis took place between 50 and 60 h during starvation.

A further interesting result was obtained using nocodazole. On one occasion the drug was added at intervals during starvation to a culture which did not sporulate after illumination at 72 h but only sporulated when illuminated again at 96 h. This was a culture which was beginning to lose sporulation capacity after subculture as microplasmodia for several months. This culture gave different results from normal (Table 19). In this case escape from nocodazole inhibition of sporulation did not occur until between 72 and 90 h during starvation, at least 24 h later than it occurred in the good sporulating strain (Table 18). This suggests that a function inhibited by nocodazole must occur during the last 24 h of starvation before a plasmodium can respond to light and eventually sporulate.

Table 18 Effect of nocodazole on sporulation

Filter papers supporting starving plasmodia were transferred from SM to SM + 20 μgml^{-1} nocodazole at times during starvation and the effect on sporulation frequency after illumination at 72 h noted

Time (h) of addition of nocodazole during starvation	Sporulation Frequency				Total
	Expt 1	Expt 2	Expt 3	Expt 4	
0	0/2	0/2	0/4	0/2	0/10
24	0/2	0/2	0/4	0/2	0/10
48	0/2	0/2	2/4	0/2	2/10
50	-	0/2	2/4	0/2	2/8
55	-	2/2	3/4	1/2	6/8
65	-	-	3/4	1/2	4/6
72	2/2	2/2	4/4	2/2	10/10
74	-	2/2	-	2/2	4/4
control (no addition)	2/2	2/2	4/4	2/2	10/10

Table 19

Effect of nocodazole on a culture exhibiting delayed sporulation

The experiment was carried out as for Table 18. The culture used was beginning to lose the capacity for sporulation and was not sporulating after illumination at 72 h, but did so after further illumination at 96 h.

Time (h) of addition of nocodazole during starvation	Sporulation frequency	
	After illumn. at 72 h	After illumn. at 96 h
24	0/4	0/4
48	0/4	0/4
50	0/4	0/4
65	0/4	0/4
72	0/4	0/4
90	0/4	4/4
control (no addition)	0/4	4/4

ii) Inhibitors of DNA synthesis

Initially it was proposed to use the inhibitors of DNA synthesis, AraC (cytosine-1- β -D-arabinofuranoside) and 5-bromo-2'-deoxyuridine (BUdR) (an analogue of thymidine which interferes with DNA replication at high concentrations). AraC was found to have very little effect on growth of microplasmodia even at high concentrations (Fig 10). Consequently hydroxyurea was tested as an alternative. Hydroxyurea could prevent growth of microplasmodia at a concentration of 500 μgml^{-1} (Fig 10). BUdR at concentrations of 100 μgml^{-1} and more had a marked effect on growth of microplasmodia (Fig 11) but concentrations of 25 $\mu\text{g ml}^{-1}$ and less had no effect (Fig 11).

The effect of these drugs on sporulation was tested. Hydroxyurea (500 μgml^{-1}) prevented sporulation if added prior to 24 h during starvation (Table 20). When added later than 24 h plasmodia began to escape the effect of the drug and finally sporulated normally. When a range of concentrations of BUdR were added to sporulating cultures it was found that very low concentrations, as little as 10 μgml^{-1} , prevented sporulation although these concentrations had no effect on growth of microplasmodia (Fig 11). BUdR (25 μgml^{-1}) prevented sporulation if added during the first 48 h of starvation (Table 20). The results with hydroxyurea suggested that a DNA replication occurred in the region of 24 h during starvation but thereafter no further DNA synthesis necessary for sporulation took place as after this time plasmodia sporulated successfully in the presence of the inhibitor. The results with BUdR demonstrated that low concentrations of the analogue which did not affect growth and presumably therefore had little effect on DNA synthesis, did have some effect on sporulation. However, escape from this inhibition by BUdR occurred later than that of hydroxyurea at about the

Figure 10

Effect of DNA synthesis inhibitors on growth of microplasmodia

The effect of two inhibitors of DNA synthesis, AraC and hydroxyurea, on growth of microplasmodia was assayed by measurement of extracted pigment.

- Normal medium (SDM)
- SDM + 500 μgml^{-1} hydroxyurea
- SDM + 500 μgml^{-1} AraC

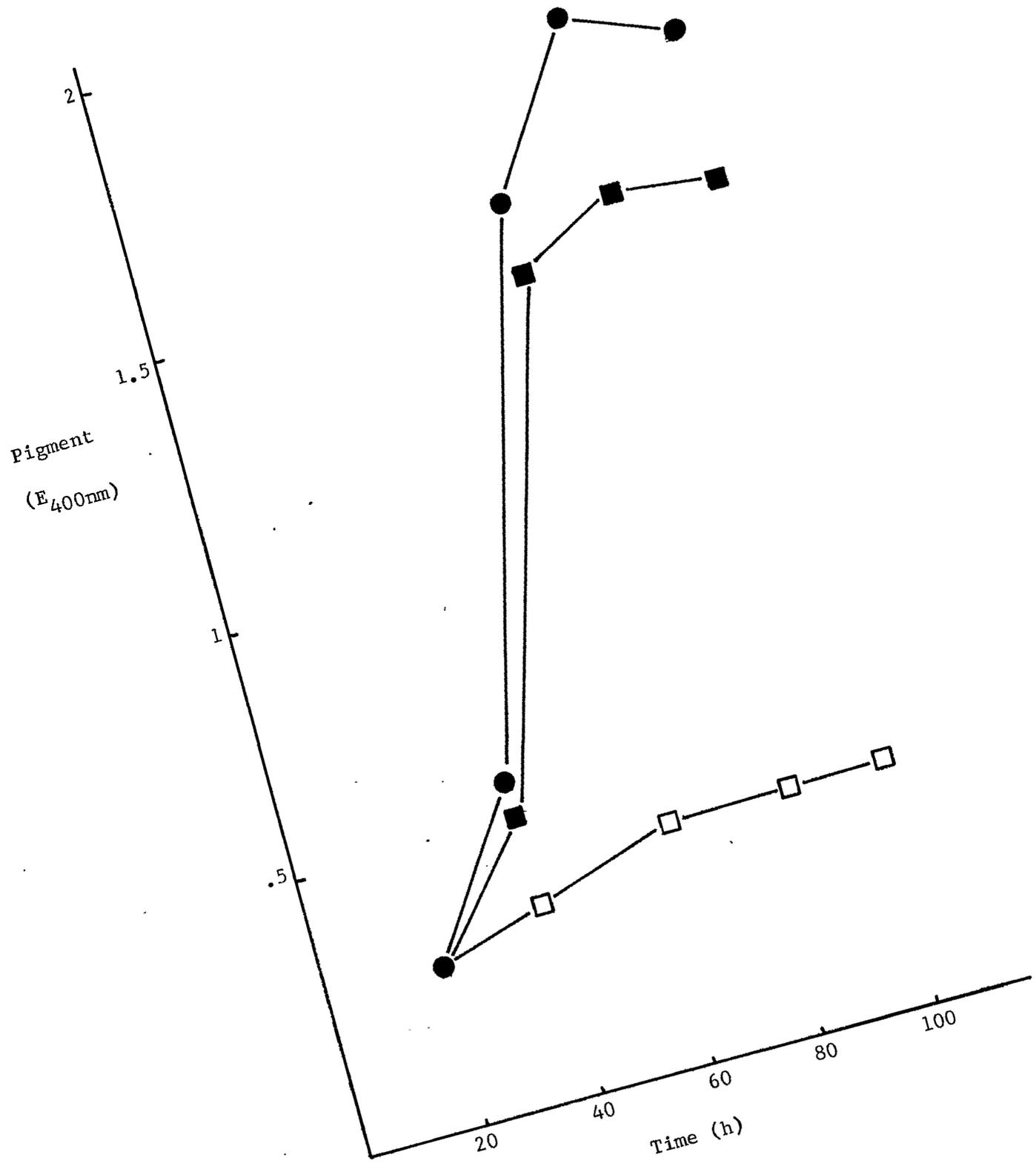


Figure 11

Effect of BUdR on growth of microplasmodia

Microplasmodia were grown in SDM and SDM + BUdR.

Growth was measured by assaying extracted pigment.

- normal medium (SDM)
- SDM + 300 μgml^{-1} BUdR
- SDM + 25 μgml^{-1} BUdR.

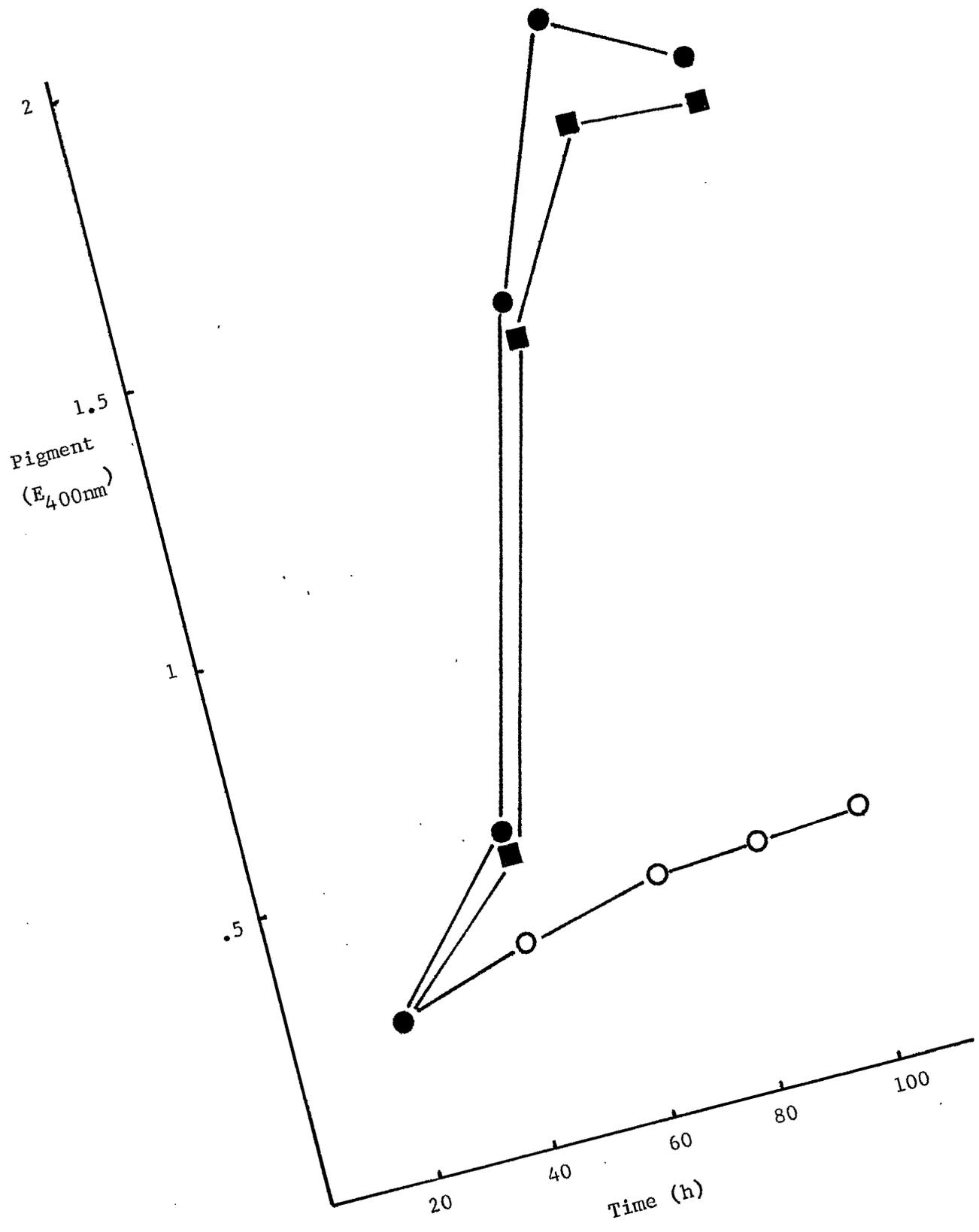


Table 20

Effect of BUdR and hydroxyurea on sporulation

Filter papers supporting starving plasmodia were transferred from SM to SM + 25 μgml^{-1} BUdR or 500 μgml^{-1} hydroxyurea at various times during starvation. Whether plasmodia sporulated normally after illumination at 72 h was recorded.

Time (h) during starvation of addition of inhibitor	Sporulation frequency	
	25 μgml^{-1} BUdR	500 μgml^{-1} hydroxyurea
0	0/6	0/10
24	0/6	2/10
48	2/6	8/10
72	6/6	10/10
control (no addition)	6/6	10/10

same time as plasmodia escaped nocodazole inhibition of sporulation (Table 18). This effect of BUdR in inhibiting differentiation at concentrations that have little effect on growth has been noted in other differentiating systems (see Discussion).

The results with inhibitors seemed to suggest that DNA replication and mitosis were necessary events during starvation if sporulation was to occur. Results with nocodazole suggested that mitosis occurred during starvation between 48 h and 72 h, although this assumes that the effect of the inhibitor was on the function of the microtubules of the spindle and not any other microtubules (see Discussion). Mitoses prior to this time could also have occurred, but these would not be identified by use of the inhibitor. If a mitosis did occur between 48 and 72 h then it would be important for two reasons. First it did not seem to be followed by DNA replication as plasmodia exposed to hydroxyurea at this time sporulated normally; the pattern of DNA synthesis during starvation is reported below. Second, it appeared to play an important role in determining when the starved plasmodia became capable of responding to light, as a delay in the occurrence of this mitosis resulted in a delay of the time at which light would induce sporulation.

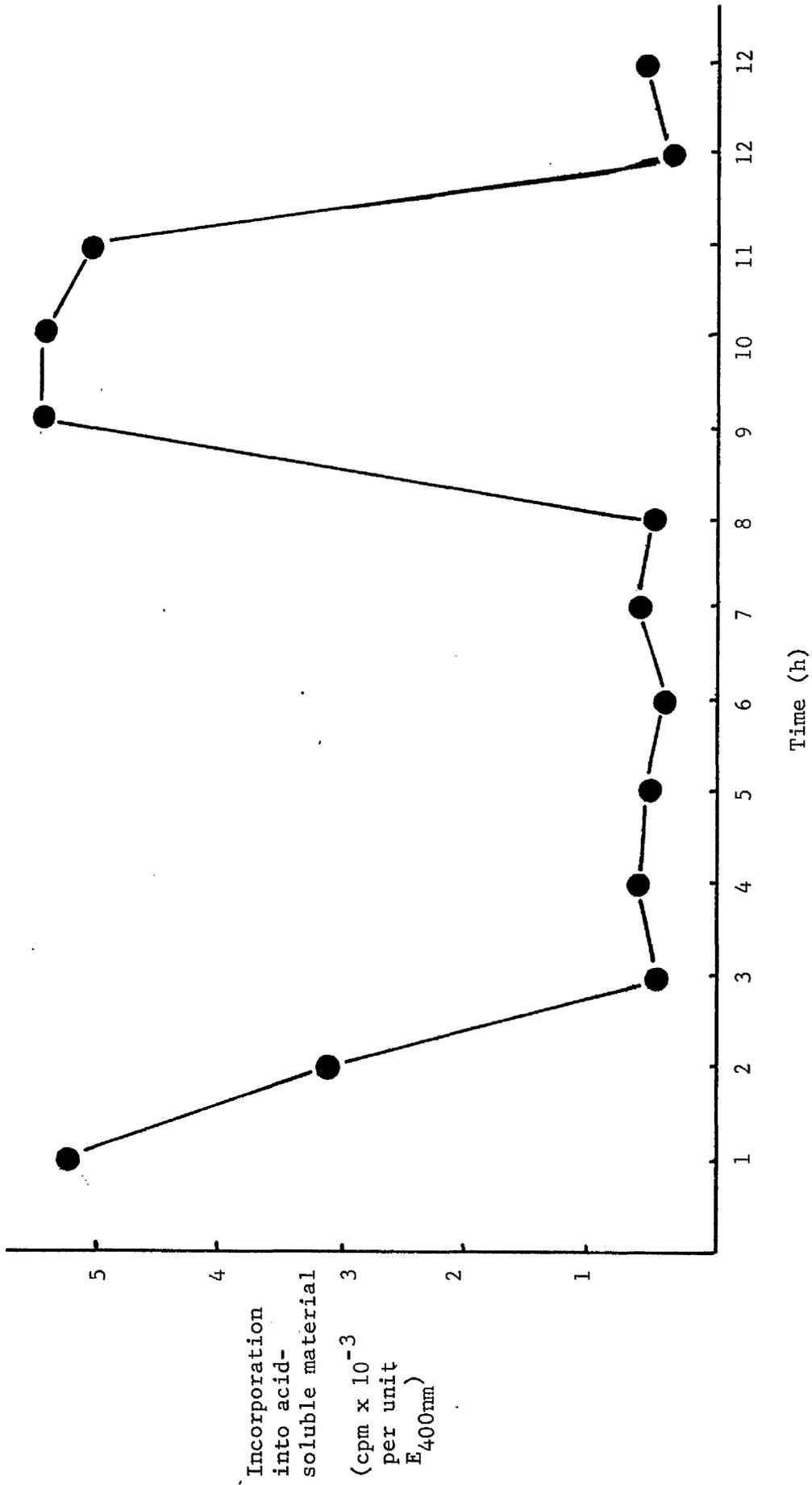
8 DNA synthesis in growing and starving plasmodia

Incorporation of [methyl-³H] thymidine into acid insoluble material by growing and starving plasmodia was studied. In a growing plasmodium a peak of uptake of [³H] thymidine lasting 2 to 3 h could readily be demonstrated (Fig 12); approximately 8 h separated one peak from the next. These peaks of incorporation of [³H] thymidine associated with DNA replication occur immediately after mitosis in P. polycephalum since it has no distinguishable G1 period in its cell

Figure 12

DNA synthesis in a growing plasmodium

Uptake of [methyl-³H]thymidine into a growing plasmodium (24-48 h old) was examined. Pieces of filter supporting the plasmodium were cut out with scissors and transferred to radioactive medium at hourly intervals. After 30 min on the radioactive medium pieces of plasmodium were plunged into TCA acetone which removed all pigment from the plasmodium. The final radioactive count was standardised to 1 unit of $E_{400\text{nm}}$ for each piece of plasmodium.



cycle (Nygaard et al, 1960; Braun et al, 1965). The pattern of incorporation was shown to be unaffected by excess uridine in the medium (Fig 13) and it was assumed that incorporation of label into RNA was not affecting results. In order to clarify further the effect of the inhibitors used in the preceding section their effect on uptake of [^3H] thymidine into growing plasmodia was investigated (Fig 14). AraC the inhibitor which did not prevent growth of microplasmodia also had no effect on [^3H] thymidine incorporation into plasmodia whilst hydroxyurea completely prevented it (Fig 14a). The mitotic inhibitor nocodazole had no effect on DNA synthesis. The high concentration of BUdR prevented DNA synthesis (Fig 14b) whilst the low concentration, although it did not prevent incorporation, did reduce the level of incorporation, probably by direct competition for uptake with thymidine. The low concentration of BUdR, which prevented sporulation (Table 20), would not therefore appear to be doing so by directly affecting DNA replication.

Since experiments with hydroxyurea had suggested that a replication of DNA occurred between 24 h and 48 h during starvation it was decided to attempt to find this and any other peaks of incorporation of [^3H] thymidine which might occur during starvation. Only one peak of incorporation was observed between 20 and 50 h (Fig 15), this occurred between 22 and 25 h. In four experiments using separate plasmodia a peak of uptake of [^3H] thymidine was observed between 20 and 24 h during starvation (Fig 16). Of the four plasmodia used in these experiments b and c were prepared from the same microplasmodial subculture whilst a and d were prepared from different cultures (Fig 16). It can be seen that in surface plasmodia derived from different liquid subcultures of microplasmodia there was some variation in the time of onset of the period of DNA replication, but in plasmodia prepared from a single

Figure 13

Effect of excess uridine on uptake of [methyl-³H]
thymidine in growing plasmodia

Uptake of [³H] thymidine into a 24 h old growing plasmodium was measured as in figure 13. At each time of sampling one piece of plasmodium was placed on normal radioactive medium and a second piece was placed on radioactive medium containing 100 µgml⁻¹ uridine.

- growing plasmodium, uptake of [³H] thymidine
- growing plasmodium, uptake measured in the presence of excess uridine.

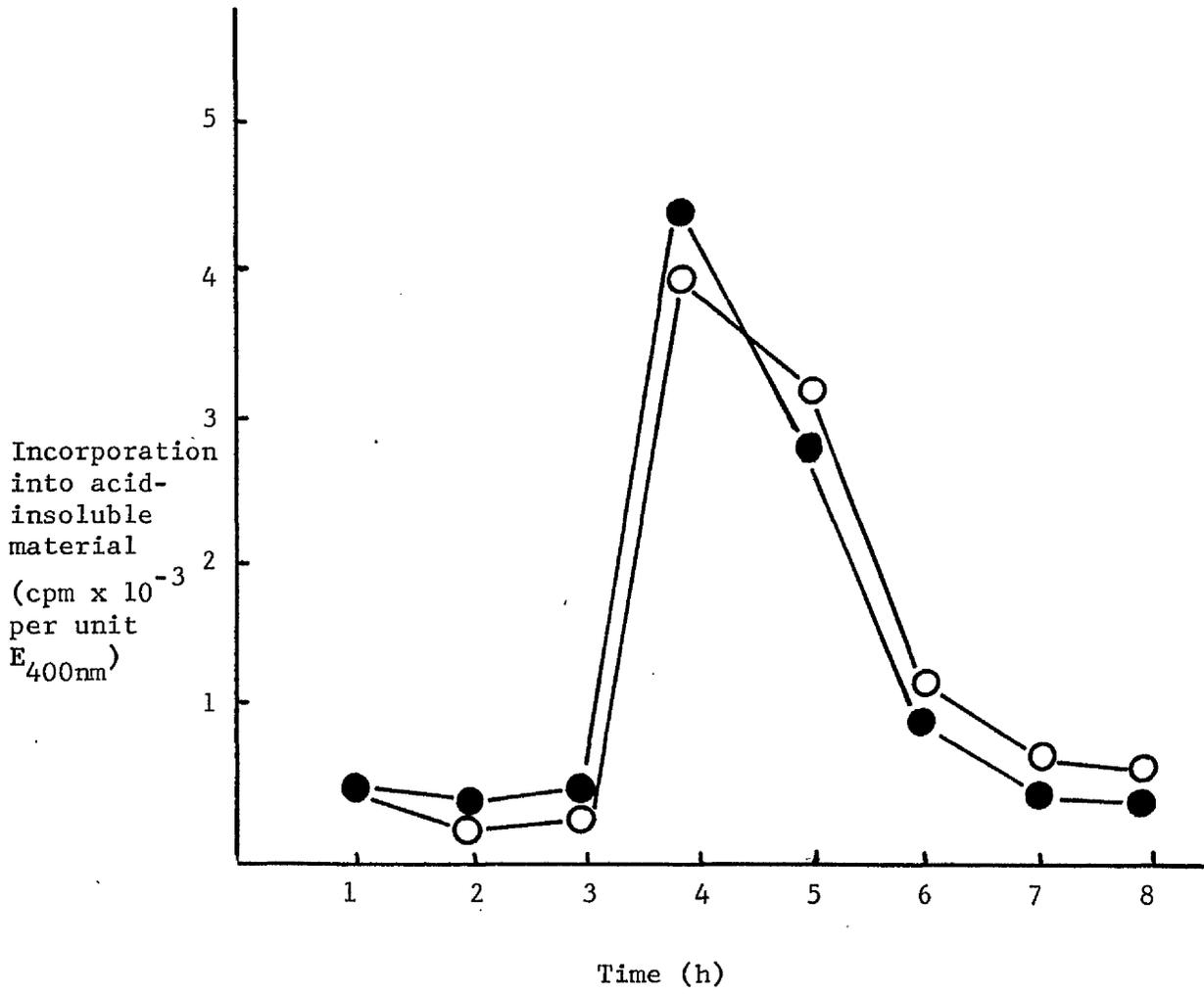


Figure 14

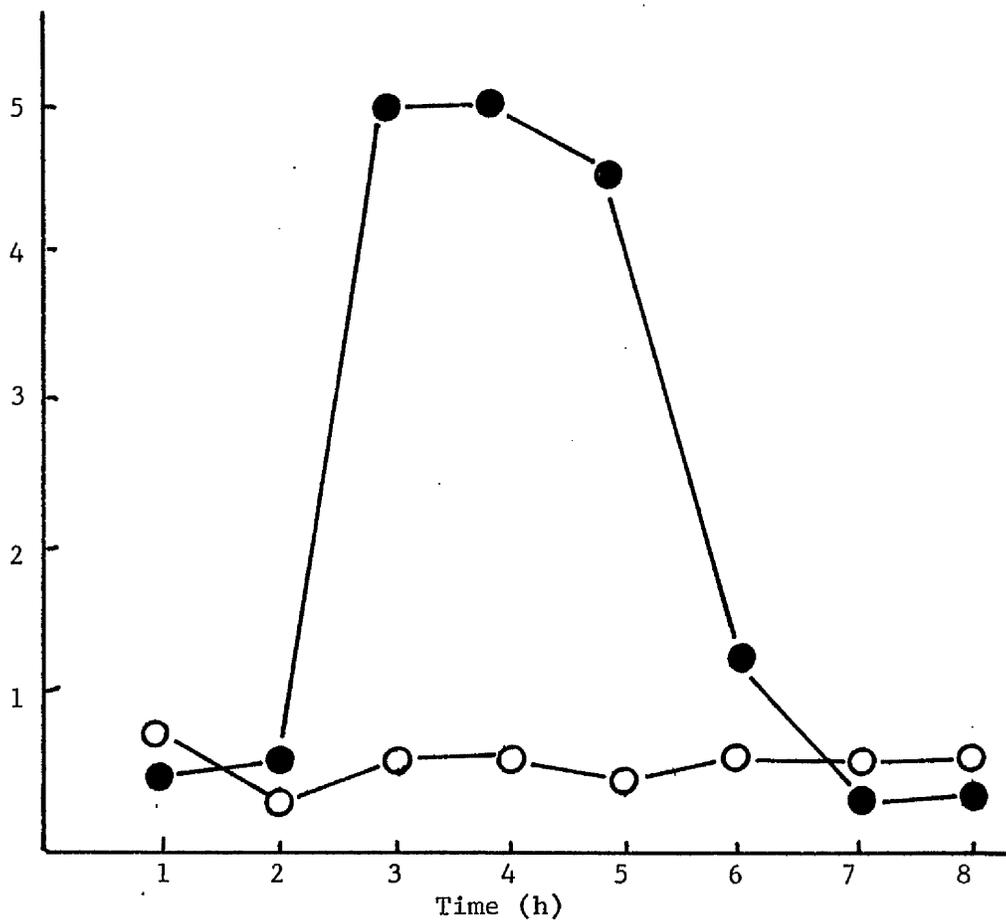
Effect of inhibitors on DNA synthesis in growing plasmodia

Uptake of $[^3\text{H}]$ thymidine into growing plasmodia was measured as in fig 13. At each time of sampling two pieces of plasmodium were removed, one placed on normal radioactive medium and the other on radioactive medium containing the inhibitor being studied.

- a) ● example of result obtained from normal growing plasmodia and plasmodia transferred to radioactive medium containing either $500 \mu\text{gml}^{-1}$ AraC or $20 \mu\text{gml}^{-1}$ nocodazole
- example of result obtained from plasmodia transferred to radioactive medium containing $500 \mu\text{gml}^{-1}$ hydroxyurea
- b) ● normal incorporation
- $300 \mu\text{gml}^{-1}$ BUdR
- $25 \mu\text{gml}^{-1}$ BUdR

a)

Incorporation into acid-insoluble material
(cpm x 10⁻³ per unit E_{400nm})



b)

Incorporation into acid-insoluble material
(cpm x 10⁻³ per unit E_{400nm})

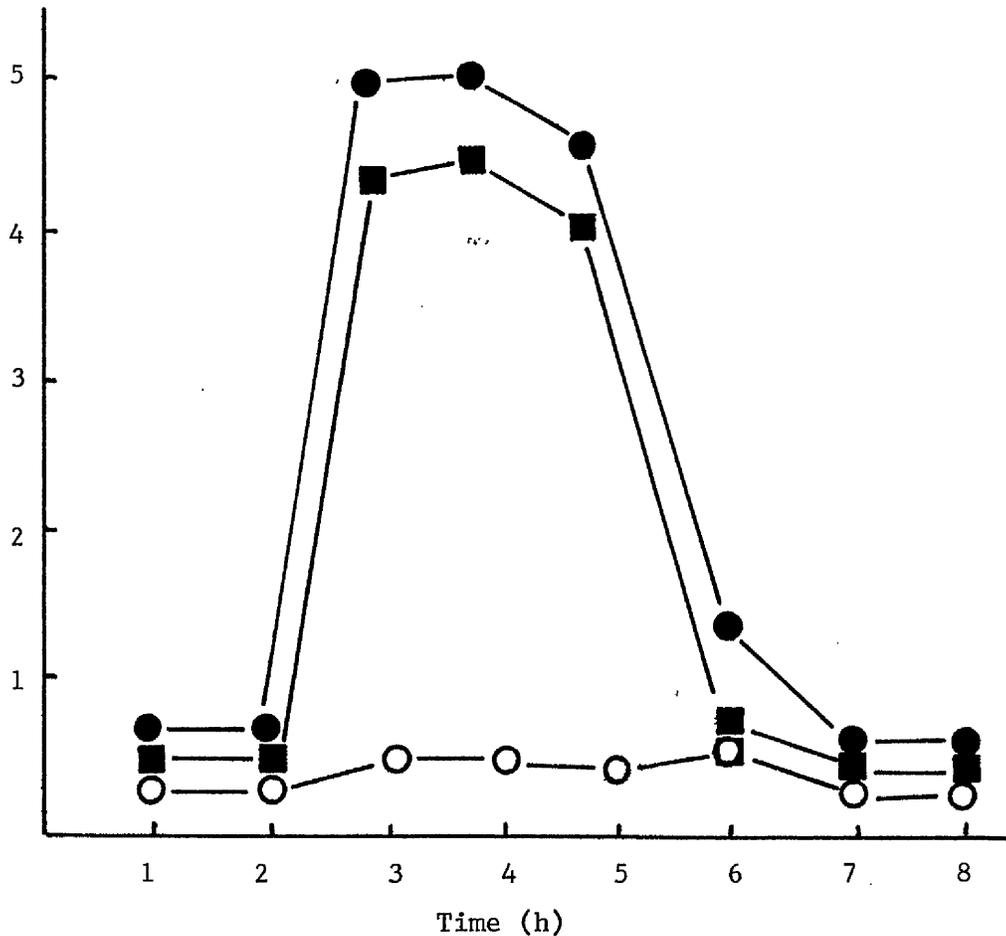
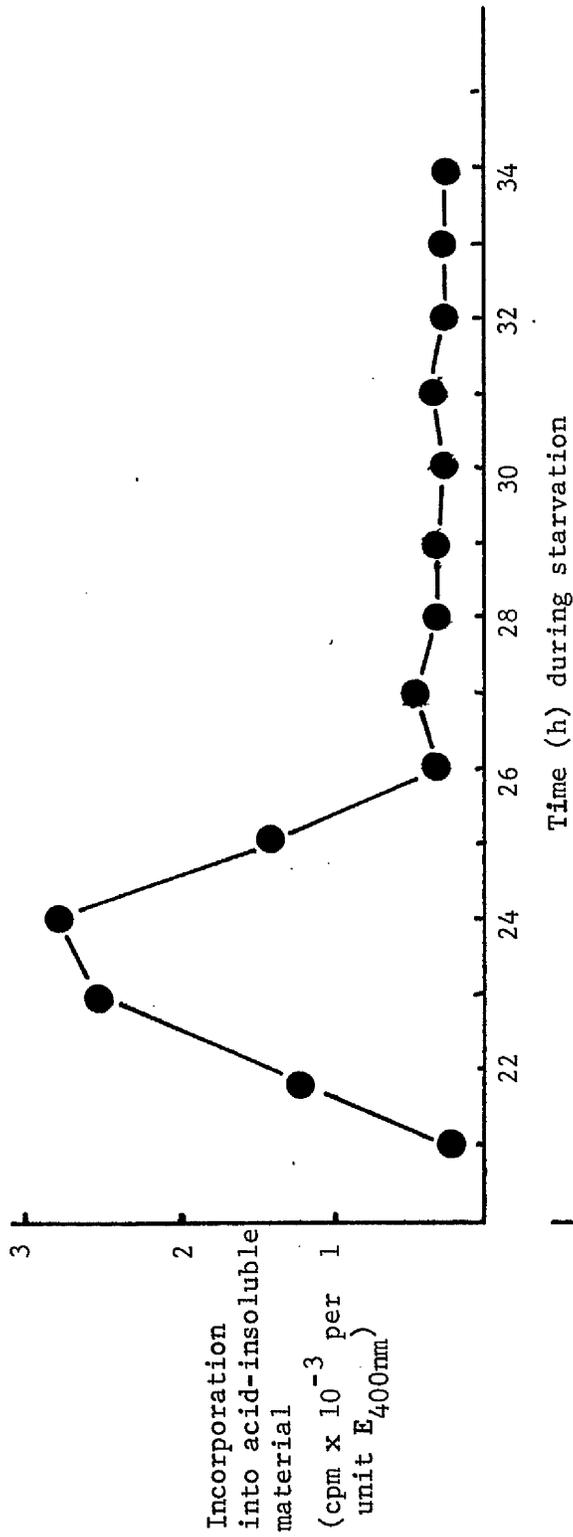


Figure 15

DNA synthesis between 20 and 48 h during starvation

Uptake of [^3H] thymidine into starved plasmodia was measured as in fig 13. Plasmodia were starved on filter papers as described in Methods section 1e. a & b represent results obtained from 2 separate plasmodia prepared on different days.

a)



b)

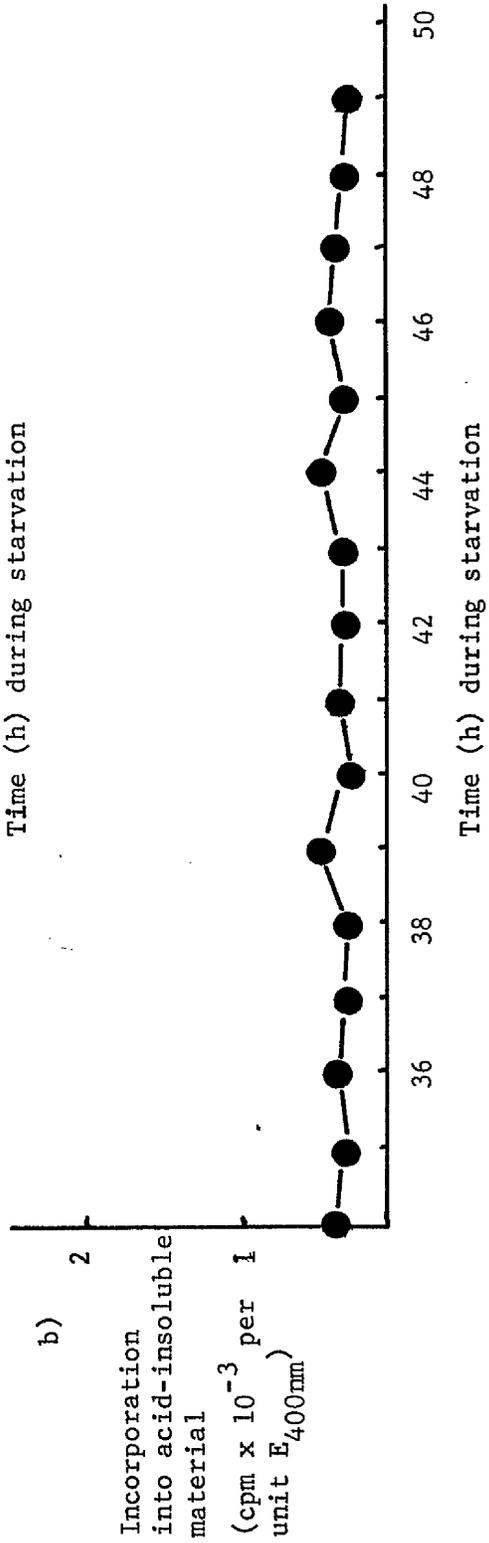
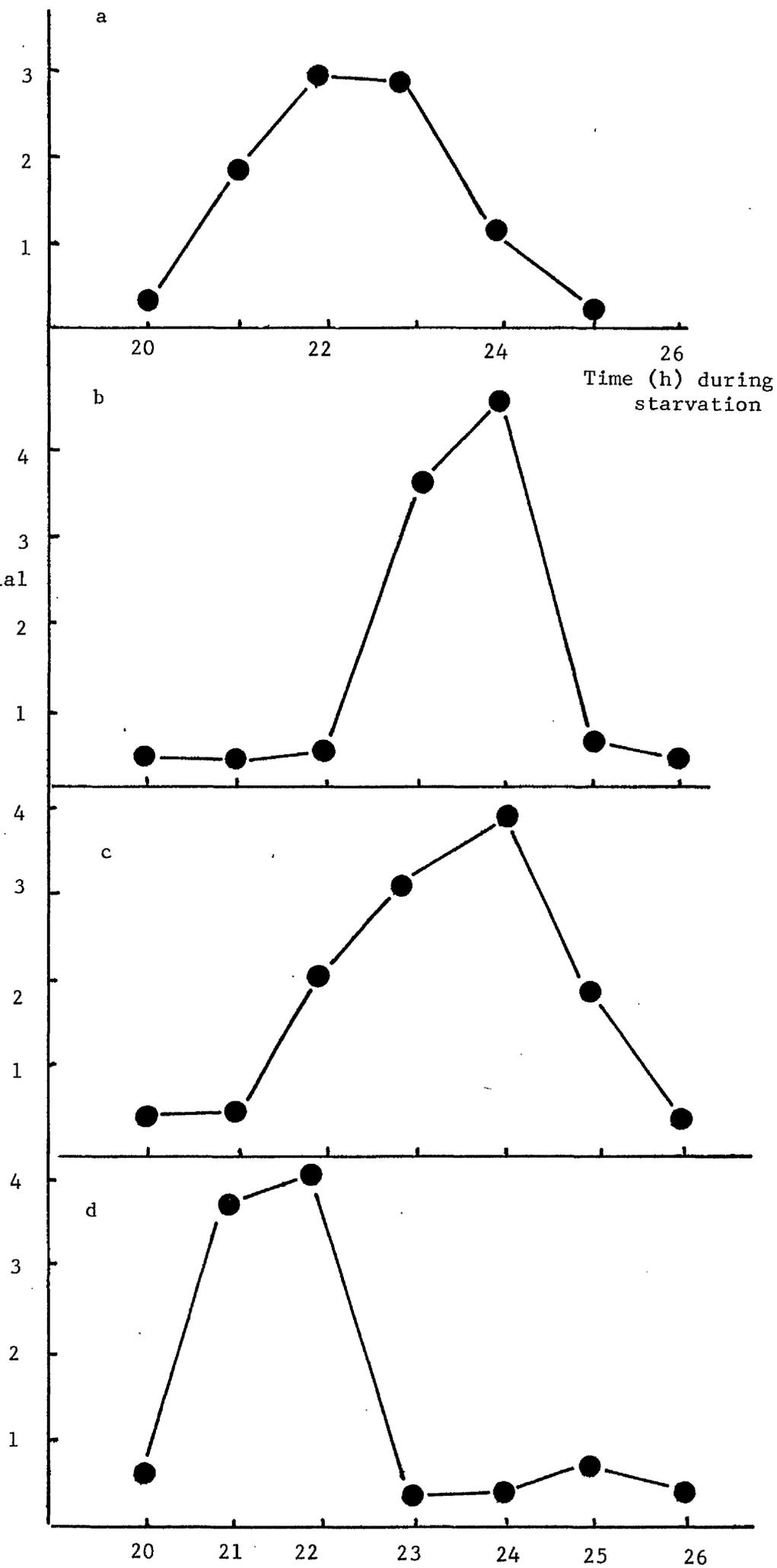


Figure 16

DNA synthesis in starving plasmodia

Uptake of [^3H] thymidine into starved plasmodia, prepared as in Methods section 1e, was measured as in fig 13. Of the four plasmodia sampled, b and c were prepared from the same microplasmodial culture whereas a and d were prepared from separate microplasmodial cultures. After all samples had been removed from a filter paper the small piece remaining (generally about 2 cm^2) was illuminated at 72 h; cultures a to d all sporulated after illumination at 72 h.

Incorporation
into acid-
insoluble material
(cpm $\times 10^{-3}$
per unit
 E_{400nm})



population of microplasmodia DNA replication began in almost perfect synchrony (Fig 16b,c). Mitotic synchrony in individual growing plasmodia prepared from a single culture of microplasmodia was noted by Guttis et al, (1961).

When plasmodia from the normal sporulating strain were compared with plasmodia formed from a strain which had become asporogenous after repeated subculture (this was equivalent to subculture C in table 13 which had since been further subcultured), a peak of uptake of radioactivity was only found in the sporulating culture (Fig 17). No incorporation of radioactivity was observed in the asporogenous strain between 21 and 32 h during starvation. Consequently it seemed that the last DNA replication during starvation did occur at about 24 h in the wild type strain, as suggested by the effect of hydroxyurea on sporulation (Table 20). Furthermore it would appear that this replication must occur if plasmodia are subsequently to be capable of sporulation after illumination at 72 h. Unfortunately it was not possible to determine whether the replication at 24 h was completely missing or merely delayed beyond 32 h in the asporogenous strain as the culture being used was lost by contamination before uptake experiments later in starvation were conducted. Uptake in the sporogenous strain was not followed after 50 h during starvation but uptake between 65 and 72 h during starvation, immediately before illumination, revealed no incorporation of thymidine. Examination of DNA synthesis earlier in starvation revealed two further periods of replication 3 to 4 h after exposure of the plasmodium to SM and between 13 and 15 h (Fig 18). In these cases DNA synthesis appeared to occur normally in the asporogenous strain. The necessity for successful completion of mitosis prior to DNA replication was stressed by the fact that transfer of a

Figure 17

DNA synthesis in starving plasmodia of a sporogenous and an
asporogenous strain

Uptake of $[^3\text{H}]$ thymidine was measured during starvation as in fig 16. Sections of plasmodia remaining after all samples had been removed were illuminated at 72 h.

- culture which sporulated normally after illumination at 72 h.

- culture which did not sporulate after illumination at 72 h nor after a further period of illumination at 96 h (equivalent of subculture C in table 13 which had since been further subcultured and had become completely asporogenous).

This experiment was performed twice, the figure gives an example of the results obtained.

Incorporation
into acid-insoluble
material

(c.p.m. $\times 10^{-3}$
per unit
 E_{400nm})

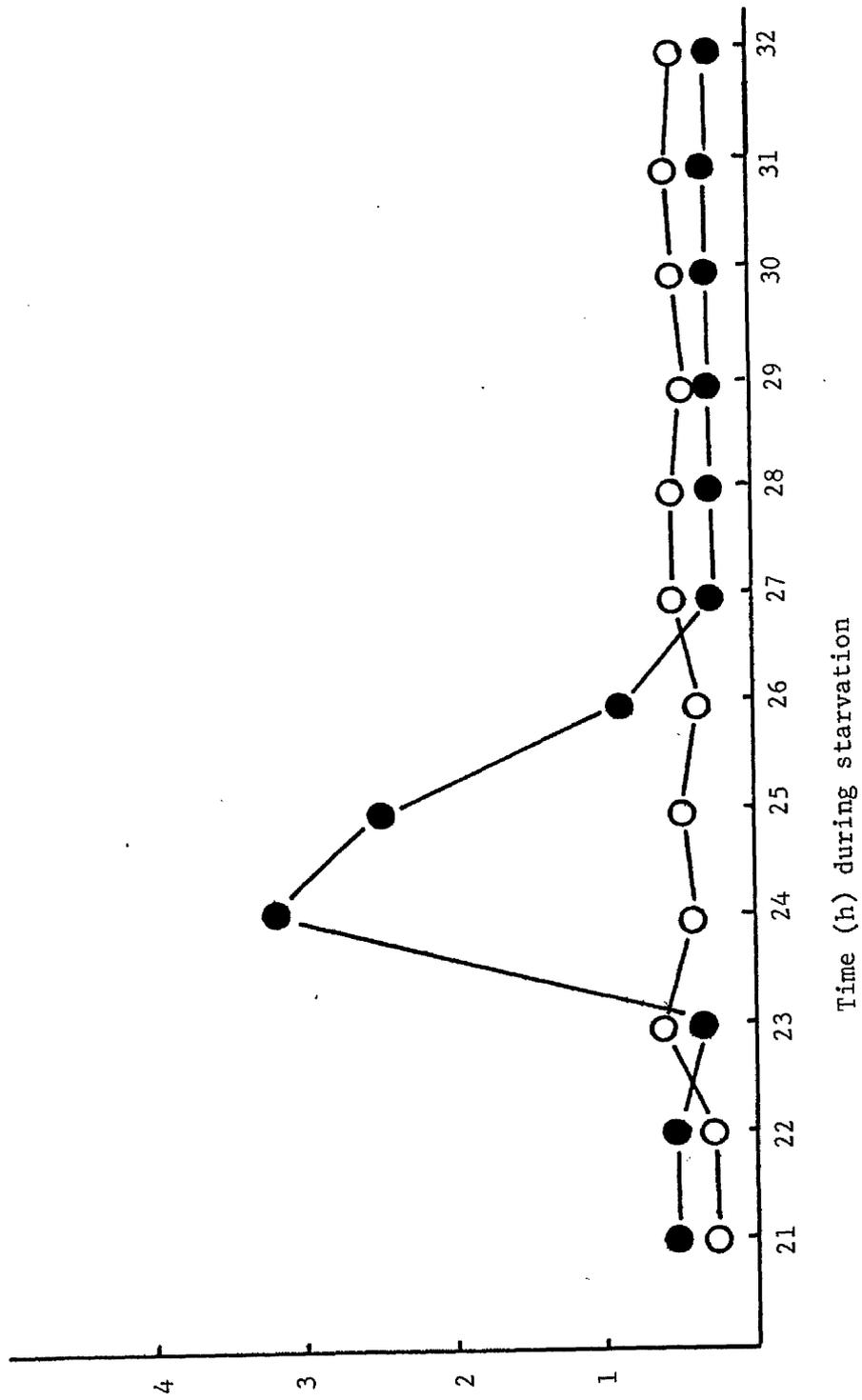


Figure 18

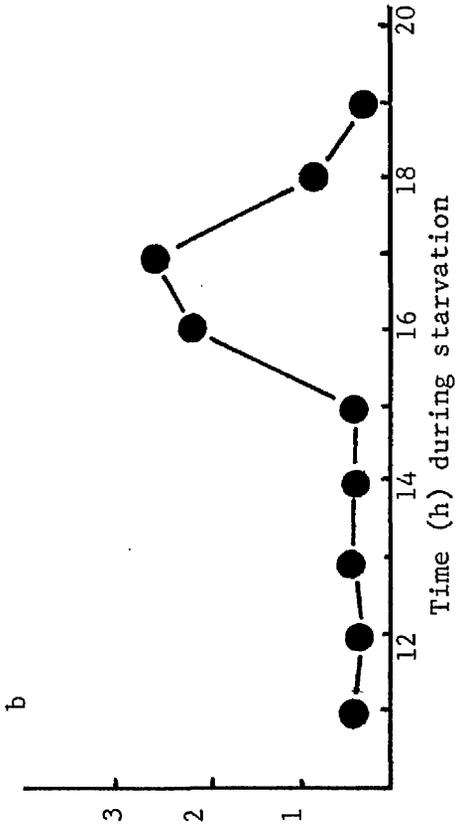
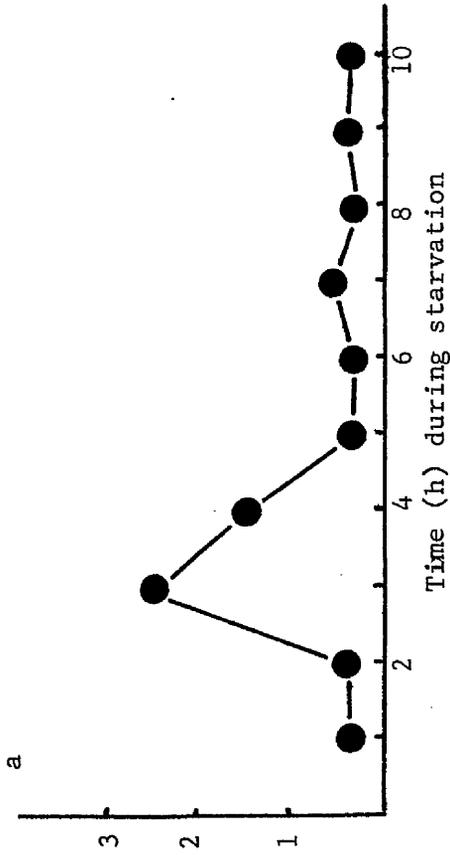
DNA synthesis during the first 20 h of starvation in a sporogenous and an asporogenous strain

Uptake of $[^3\text{H}]$ thymidine and sporulation were measured as in fig 16.

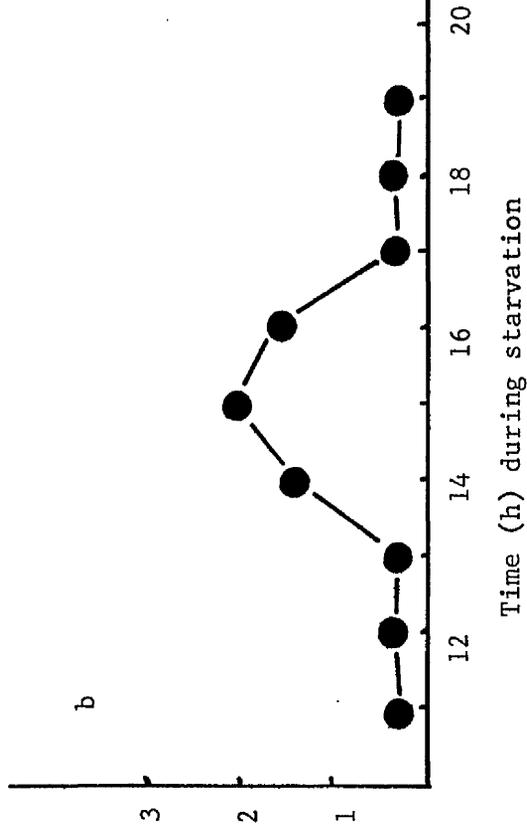
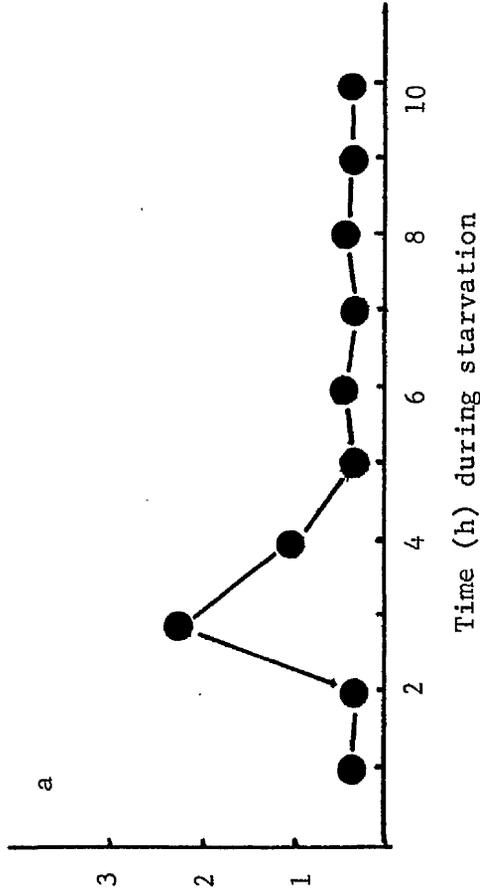
1. sporogenous culture
2. asporogenous culture (equivalent of subculture C in table 13 which had since been further subcultured).
a and b in each case represent results obtained from 2 different plasmodia prepared on different days.

This figure represents an example of the results obtained; the experiment was conducted twice and results were the same on each occasion with only slight variations in the times at which each replication began.

Incorporation
into acid-
insoluble
material
(cpm x 10⁻³
per unit
E_{400nm})



Incorporation
into acid-
insoluble
material
(cpm x 10⁻³
per unit
E_{400nm})



starving or a growing plasmodium to medium containing nocodazole between rounds of DNA replication abolished the following period of DNA synthesis (Fig 19).

9 Stability of inhibitors in microplasmodial cultures and development of resistance of the organism to inhibitors

The fact that many inhibitors commonly used in other systems have no effect on P. polycephalum may be related to impermeability of the plasmodium to inhibitors. Noticeably AraC as a DNA replication inhibitor failed to prevent growth of microplasmodia of P. polycephalum (Fig 10). Changes in permeability to an inhibitor must be considered as a possible explanation for the escape of plasmodia from inhibition of sporulation (see Discussion). Furthermore, during the course of this work changes in response to inhibitors by Physarum were noted. In some early experiments the effect of the protein synthesis inhibitor cycloheximide was tested on growth of microplasmodia. This drug was found to effectively prevent growth of microplasmodia when added at a concentration of $4 \mu\text{gml}^{-1}$ (Fig 20). This concentration also prevented sporulation if added any time prior to illumination, although its effect after illumination was not tested. It was noticed that after extended incubation of microplasmodia in the presence of the drug growth resumed and that the lag time prior to renewed growth varied directly with the concentration of the inhibitor (Fig 20). If left long enough some growth was eventually obtained even in the presence of $8 \mu\text{gml}^{-1}$ cycloheximide. It seemed likely that either the microplasmodia were becoming resistant to the drug or in some way they were breaking it down and making it ineffective. Similar results were obtained with nocodazole and hydroxyurea (see below).

It was decided to attempt to determine whether microplasmodia

Figure 19

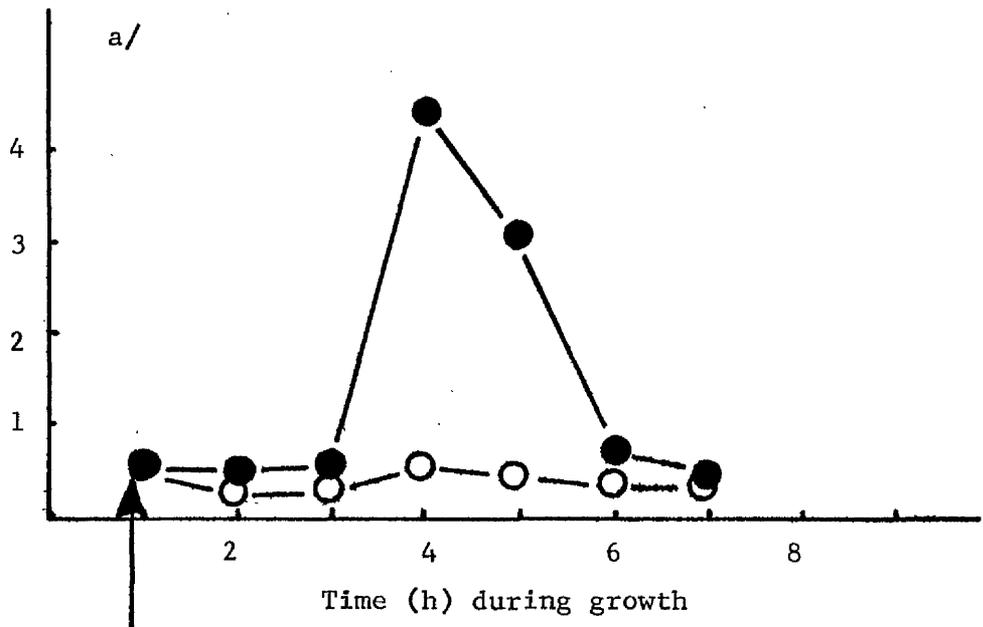
Effect of transferring plasmodia to nocodazole between
rounds of DNA replication

At the times indicated by the arrows plasmodia were halved, one half was left on normal medium and the other half transferred to medium containing $20 \mu\text{gml}^{-1}$ nocodazole. Each of the halves was sampled at hourly intervals and uptake of $[^3\text{H}]$ thymidine measured.

- a/ growing plasmodium
- b/ starving plasmodium

- normal plasmodium
- plasmodium in presence of nocodazole

Incorporation into acid-insoluble material
(cpm x 10⁻³ per unit E_{400nm})



Incorporation into acid-insoluble material
(cpm x 10⁻³ per unit E_{400nm})

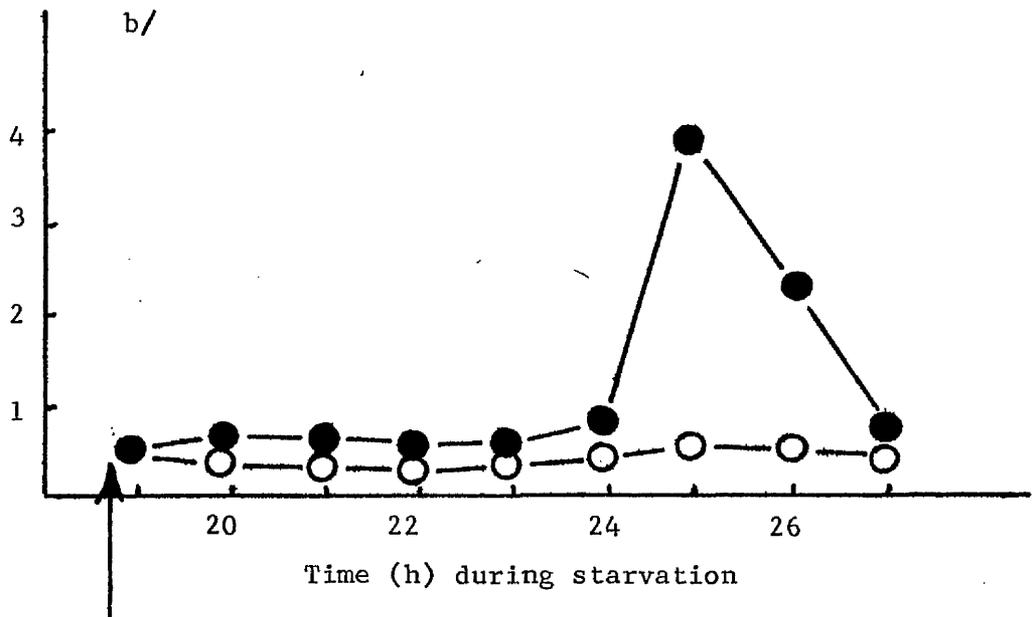
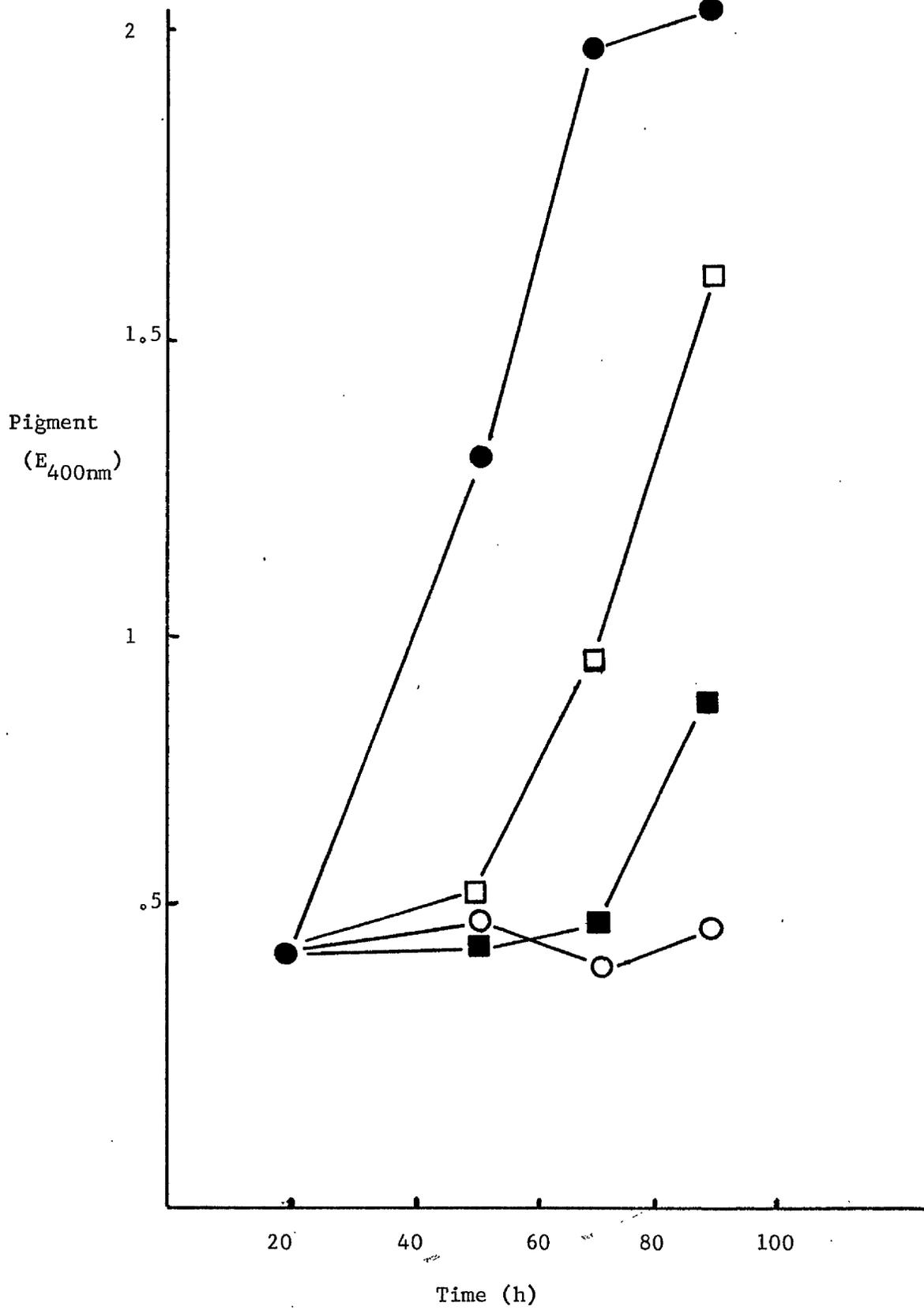


Figure 20

Growth of microplasmodia in different concentrations
of cycloheximide

Growth of microplasmodia in the presence of cycloheximide
(SDM + cycloheximide) was measured by assaying extracted
pigment.

- Normal growth
- 8 μgml^{-1} cycloheximide
- 4 μgml^{-1} "
- 2 $\mu\text{g ml}^{-1}$ "



were becoming resistant or in some way were removing the drugs from the medium. Cultures were grown for 72 to 80 h in the presence of an inhibitor. At the end of this time a sample of microplasmodia was subcultured into fresh medium with inhibitor. If the microplasmodia had become resistant a shorter lag time before growth started should be observed in this second culture. The remainder of the first culture was centrifuged (500 x g, 5 min) to remove all microplasmodia and fresh microplasmodia from a normal culture were then subcultured into this used medium. If the inhibitor had been removed from the medium then growth of the new microplasmodia should start earlier. Figures 21 and 22 demonstrate that with both cycloheximide and hydroxyurea the inhibitor appears to have been removed from the medium as it was immediately able to sustain growth of fresh microplasmodia (Fig 21b & 22b) whereas the microplasmodia from the old medium were unable to grow in the presence of fresh inhibitor (Fig 21c & 22c). With nocodazole, however, a somewhat intermediate result was obtained (Fig 23). The old medium was able to sustain growth of new microplasmodia after a lag (Fig 23b) but, in addition microplasmodia from the original medium began to grow more quickly in the presence of the drug (compare Fig 23a and c). When using inhibitors with P. polycephalum these results suggest that problems may arise either with drug resistance or drug breakdown if cultures are in contact with the inhibitors for extended periods of time.

In the present study hydroxyurea and nocodazole were the two main drugs employed and these did prevent growth of microplasmodia and sporulation of starved plasmodia. Fortunately any interference with drug action, even by growing microplasmodia, took at least 80 h to manifest itself which meant that the periods of exposure to the drugs in experiments described previously would not be long enough to involve this complication.

Figure 21

Stability of cycloheximide in microplasmodial cultures
and development of resistance of the organism to the inhibitor

a/ Microplasmodia were grown in SDM containing $4 \mu\text{gml}^{-1}$ cycloheximide as described in Methods section 1b. After 80 h culture (a) was treated as follows. A 2 ml sample of the microplasmodia was subcultured into fresh medium containing $4 \mu\text{gml}^{-1}$ cyclohex (c). After removing all microplasmodia from culture (a) a 2 ml sample of fresh microplasmodia from a normal culture was added to this used medium (b).

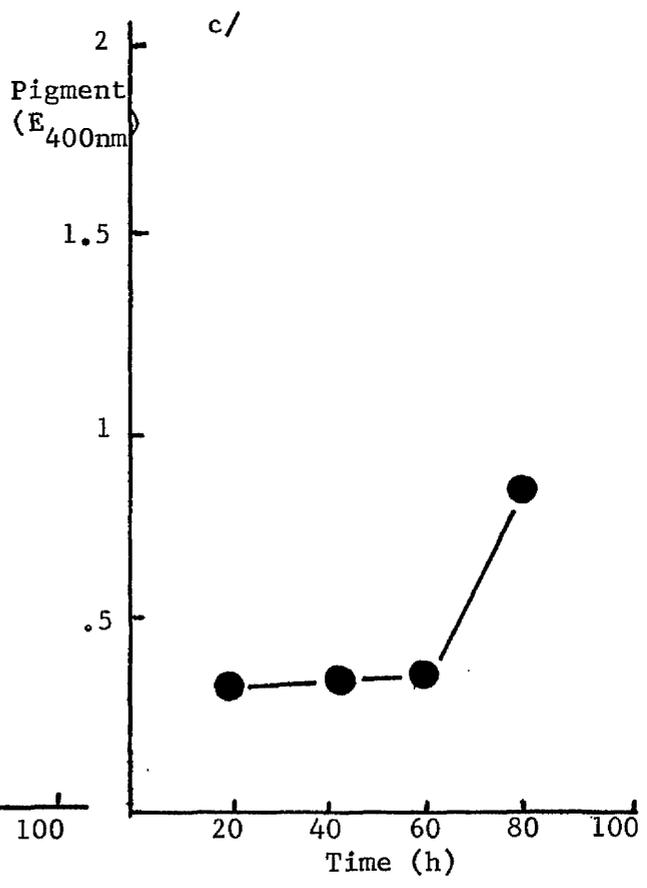
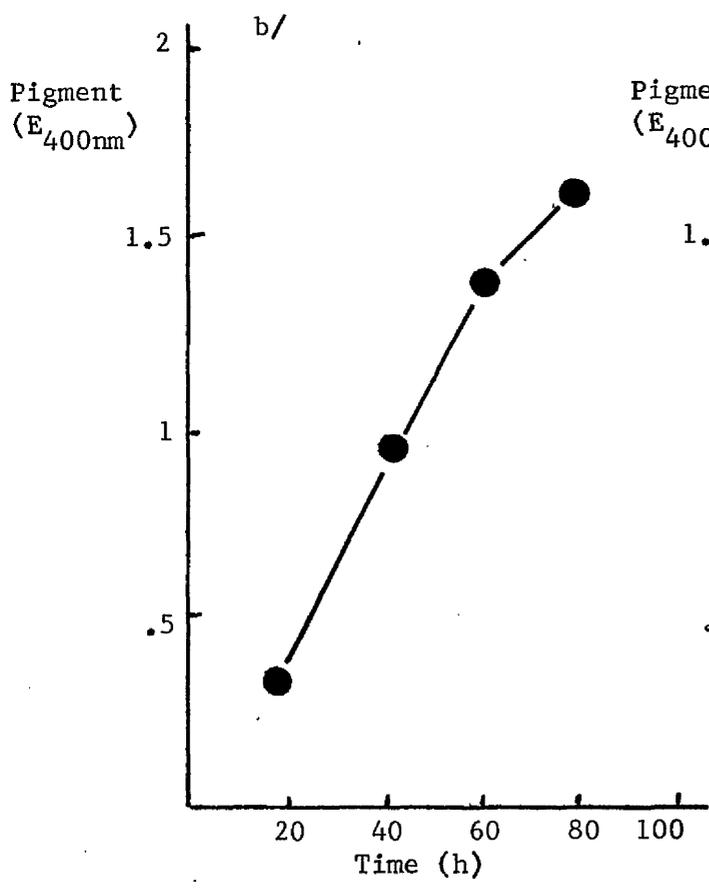
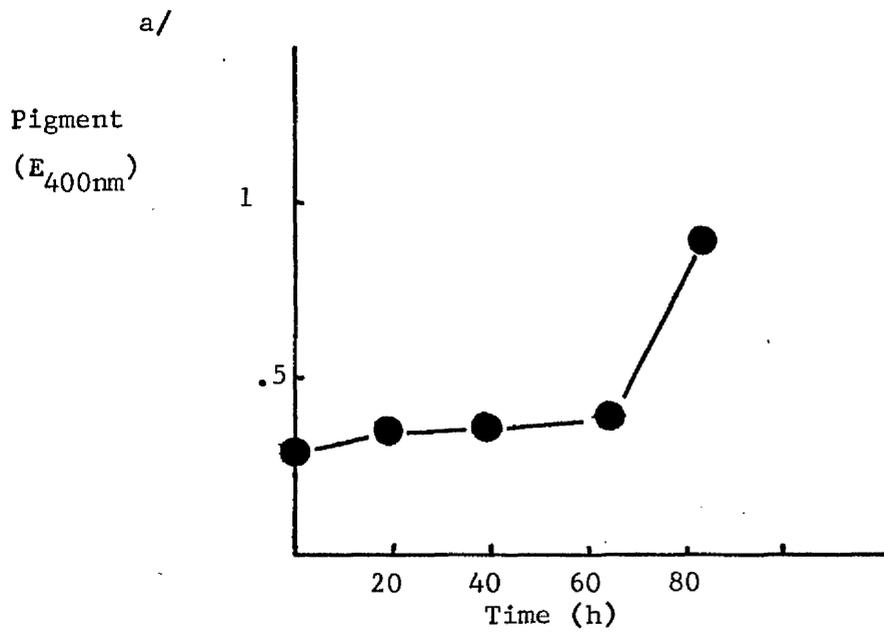


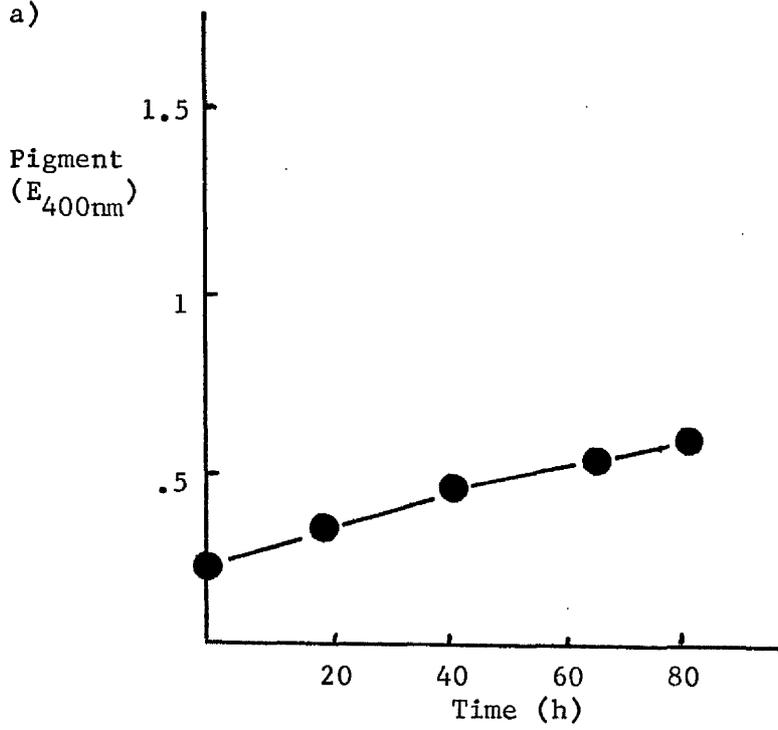
Figure 22

Stability of hydroxyurea in microplasmodial cultures and
development of resistance of the organism to the inhibitor

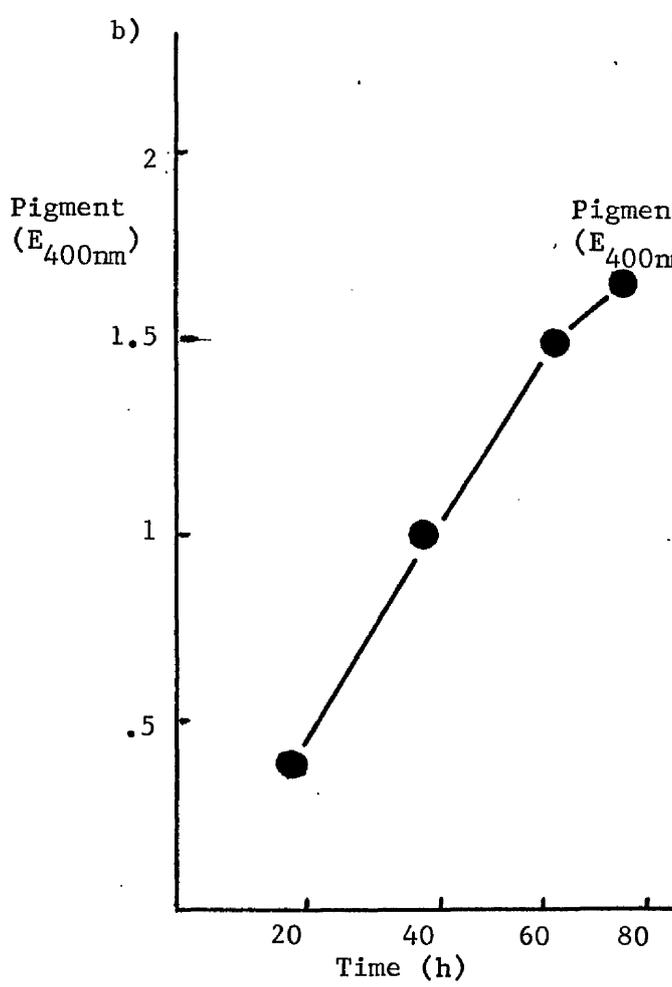
For a description of the experiment see the legend to fig 21.

- a) Growth in $500 \mu\text{gml}^{-1}$ hydroxyurea.
- b) used medium from (a) with fresh microplasmodia.
- c) microplasmodia from (a) added to fresh medium
containing $500 \mu\text{gml}^{-1}$ hydroxyurea.

a)



b)



c)

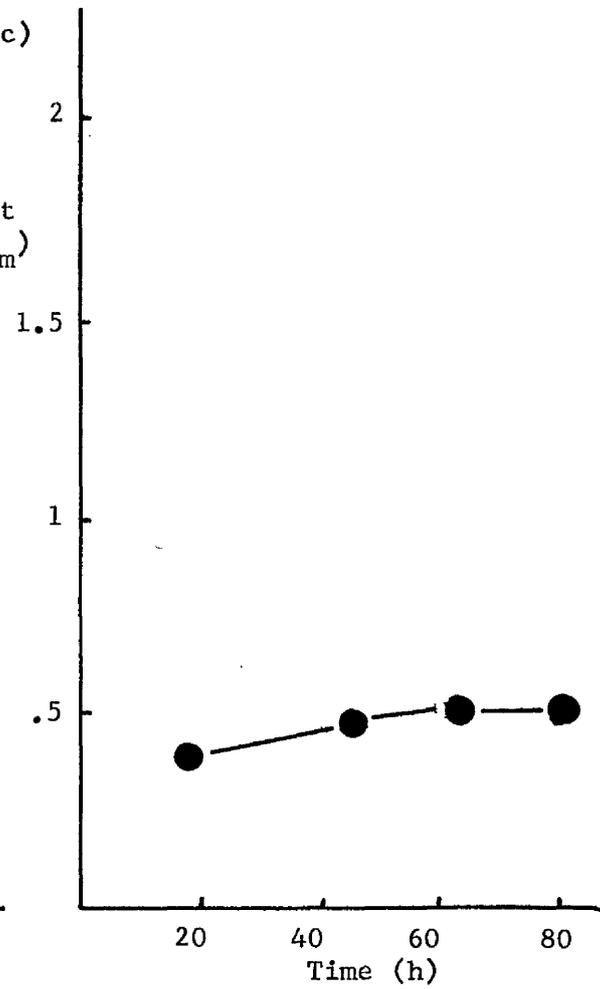
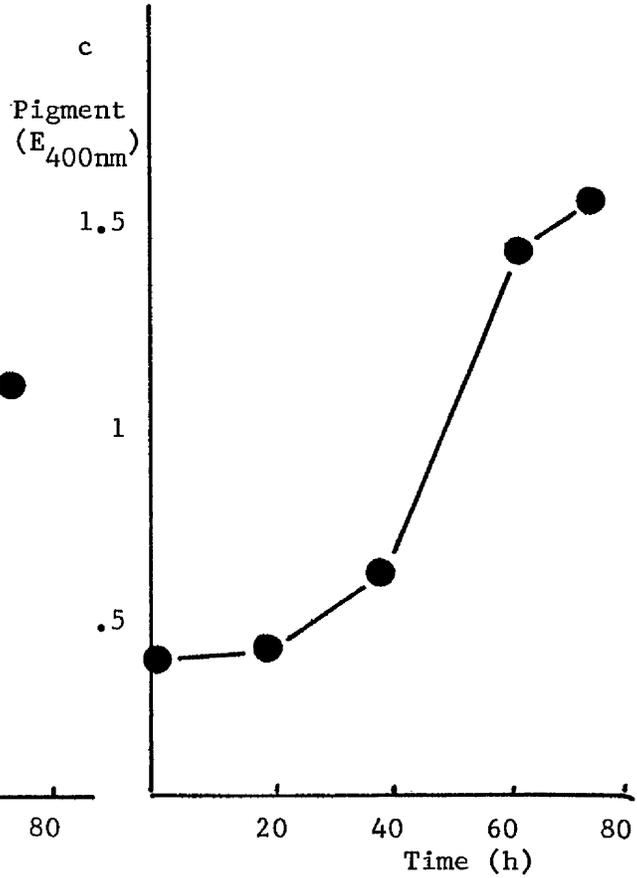
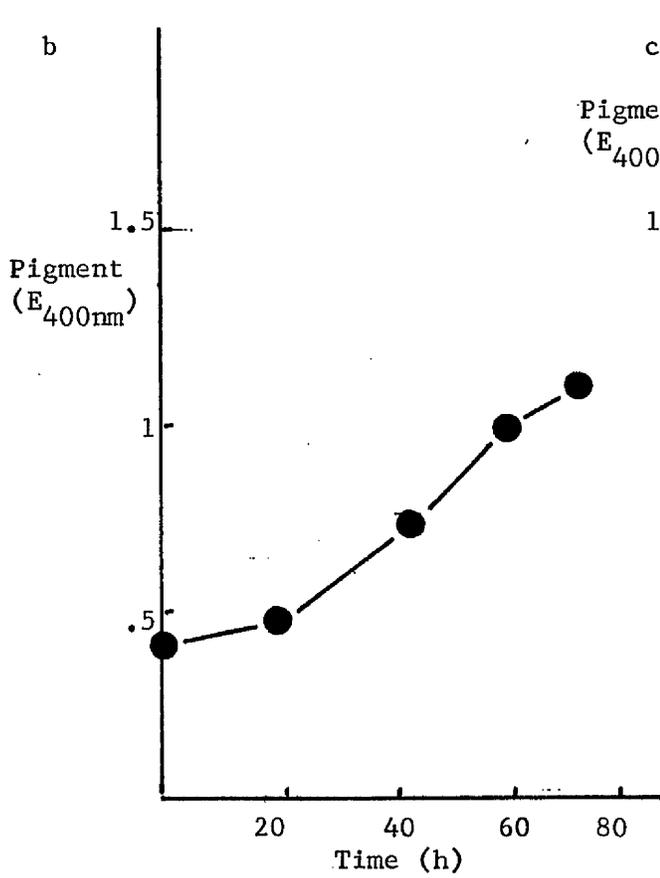
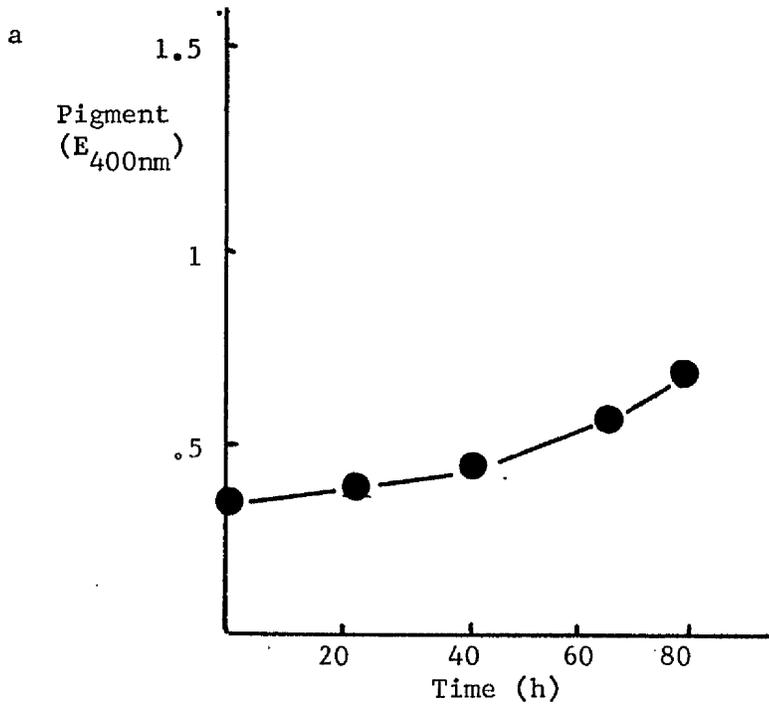


Figure 23

Stability of nocodazole in microplasmodial cultures and
development of resistance of the organism to the inhibitor

For a description of the experiment see the legend to fig 21.

- (a) Growth in $20 \mu\text{gml}^{-1}$ nocodazole
- (b) used medium from (a) with fresh microplasmodia
- (c) microplasmodia from (a) added to fresh medium
containing $20 \mu\text{gml}^{-1}$ nocodazole



10 Genetics

i) Isolation of mutants

In order to investigate further the processes involved in sporulation it would be advantageous if mutants in this process were available. An asporogenous derivative obtained by repeated subculture had already been used in the experiments on DNA synthesis, but it would be of interest to have a number of strains blocked at various stages in the process. Using these strains it should then be possible to conduct genetic analysis in order to determine if specific blocks are a result of single mutations. Consequently it should be possible to determine which events are controlled by which genes. An attempt therefore was made to mutagenise amoebae and then screen the plasmodia they gave rise to for their ability to sporulate.

N-methyl N'-nitro N-nitrosoguanidine (NTG) was used to mutagenise the amoebae. NTG at a final concentration of $250 \mu\text{gml}^{-1}$ was used and it was determined that 30 min in the presence of this concentration of NTG killed 90% of the amoebae present (Fig 24). Mutants were selected from the remaining 10% of viable amoebae. After mutagenesis amoebae required a 48 h post-mutational growth period to allow replication of the amoebae and expression of mutations (Table 21).

Initially mutants which were resistant to cycloheximide ($4 \mu\text{gml}^{-1}$) and emetine ($30 \mu\text{gml}^{-1}$) were selected in order to check that mutants could be obtained using this system. Mutants resistant to both these drugs were obtained (Table 21). Emetine resistant amoebae never arose spontaneously in control cultures, not treated with mutagen, but were obtained after mutagenesis. A cycloheximide resistant mutant was isolated on one occasion from a control culture. It was clear that, as emetine resistant mutants were only isolated after mutagenesis, the

Figure 24

Killing curve for *P. polycephalum* amoebae in 250 μgml^{-1} NTG

The number of amoebae surviving after a given exposure to NTG was calculated as in Methods section 4b, and consequently the number killed was derived.

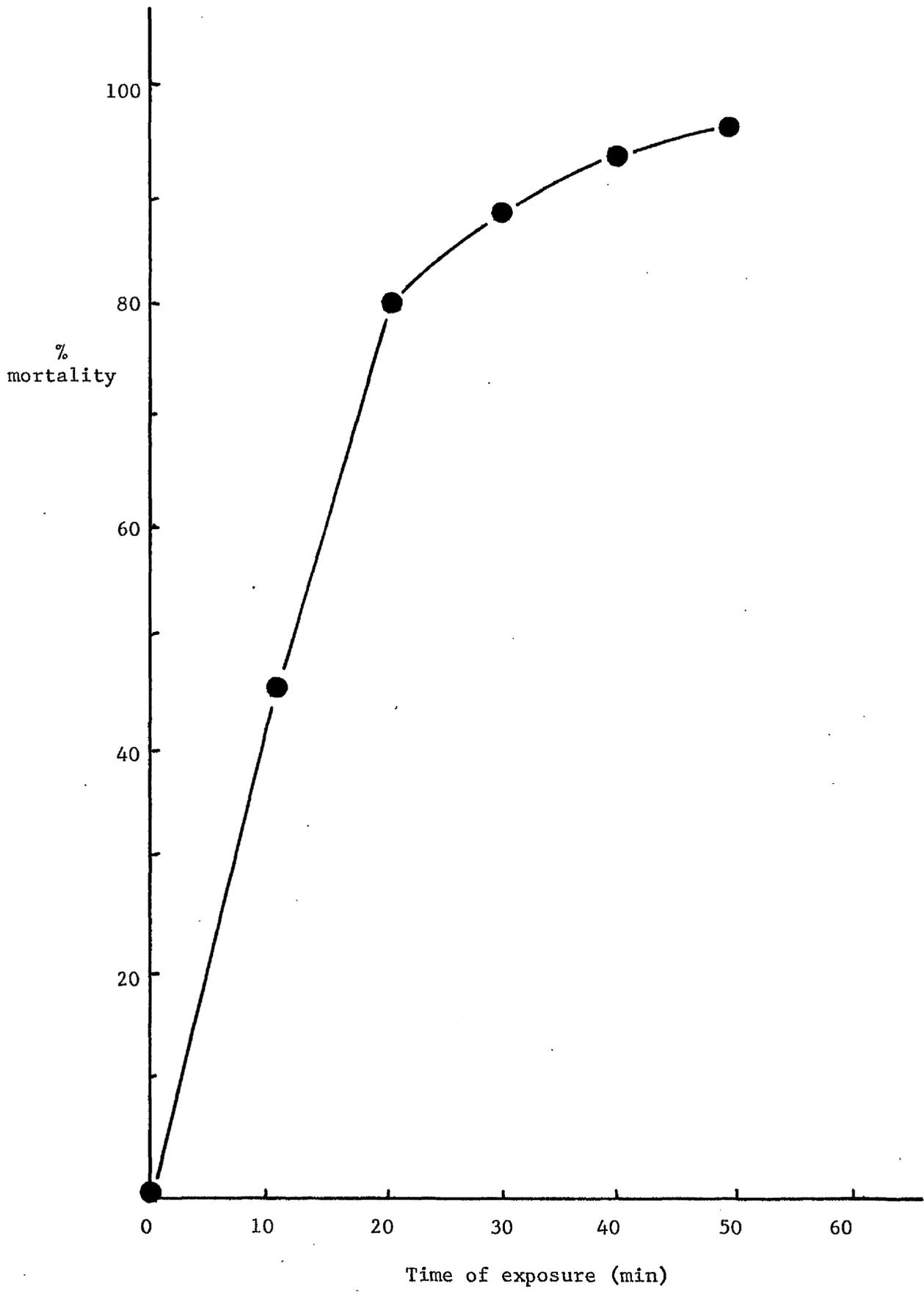


Table 21

Behaviour of amoebae in LIB after 30 min exposure to 250 μgml^{-1} NTG

After 30 min exposure to NTG samples of amoebae were washed (see Methods section 4a) and inoculated into a known volume of LIB and incubated for 0 to 48 h. At 0 h, 24 h and 48 h a range of volumes (0.05-0.15 ml) were plated onto LIA plus bacterial suspension. By counting the number of plaques obtained the number of amoebae present at each time was calculated. At the same time 0.1 ml samples were removed and plated directly onto LIA containing 4 μgml^{-1} cycloheximide or 30 μgml^{-1} emetine. Any resistant amoebae which grew on the agar were recorded.

Time (h) in LIB after exposure to NTG	No. of amoebae per ml	Mutants isolated per ml
0	1005	0
24	1560	0
48	2100	0.5 cycloheximide resistant 0.5 emetine resistant

system employed worked satisfactorily. An attempt was next made to isolate temperature-sensitive (ts) mutants which grew normally at 26°C, but grew poorly or not at all at 31°C. From such mutants it should be possible to characterise strains ts for mitosis or DNA replication as have been described previously (Wheals et al, 1976; Gingold et al, 1976; Sudbery et al, 1976; Del Castillo et al, 1978; Laffler et al, 1979; Burland & Dee, 1980). Mutants of this sort might offer an alternative means of assessing the role of these events in sporulation. Unfortunately the CL strain was unable to sporulate at 31°C and so the possibility of testing any such mutants in this way was not possible with this strain. Nevertheless, mutagenised amoebae were screened for the ts phenotype as such mutants would be useful markers for genetic analysis. In addition, plasmodia derived from them were examined for their capacity to sporulate. Seventeen amoebae were derived which showed varying degrees of inability to grow at 31°C (Table 22). None of the plasmodia derived from these amoebae showed any marked degree of temperature sensitivity, although growth of some plasmodia was poorer than normal even at 26°C (Table 22). Of these 17 ts amoebae several gave rise to plasmodia which seemed unable to sporulate (Table 22). When tested again it was confirmed that three were unable to sporulate whilst one produced abnormal sporangia which were white or pale brown and tended to form in large clumps with indistinct stalks.

It therefore seemed possible that by simply screening amoebae after mutagenesis it would be possible to isolate sporulation defective strains as quite a high percentage of the putative temperature sensitive mutants isolated produced plasmodia with defective sporulation.

A method of screening large numbers of amoebae after mutagenesis for plasmodial formation and sporulation would be advantageous

Table 22

Isolation of temperature sensitive mutants of *P. polycephalum* CL

Amoebae were mutagenised by exposure to $250 \mu\text{gml}^{-1}$ NTG for 30 min. After a 48 h post-mutational growth period amoebae were selected which grew normally at 26°C , but poorly or not at all at 31°C (see Methods section 4d). Plasmodia derived from these amoebae were also tested for their ability to grow at 26°C and 31°C and for their sporulation capacity.

Mutant No.	Amoebae		Plasmodia		Sporulating frequency at 26°C
	26°C	31°C	26°C	31°C	
1	+++	+	++	++	4/4
2	+++	+++	++	++	4/4
3	+++	+	++	+	0/4
4	+++	+	++	++	4/4
5	+++	+	++	++	4/4
6	+++	++	++	++	4/4
7	+++	+	+	++	4/4
8	+++	+	++	++	0/4
9	+++	+	++	++	4/4
10	+++	+	+	++	0/4
11	+++	+	+	++	4/4
12	+++	++	++	+	2/4
13	+++	+	+	+	0/4
14	+++	+	+	+	4/4
15	+++	+	++	++	4/4
16	+++	++	++	++	3/4
17	+++	+	++	++	2/4
wild type	+++	+++	+++	+++	4/4

for this purpose and an attempt was made to screen amoebae in multi-well tissue culture trays (see Methods section 4e(i)). Several difficulties were encountered using these trays. In all cases dehydration of the agar was a problem even when one or more of the wells in the tray were filled with water to try and increase humidity during incubation. If DSDM agar was used in the wells amoebae did form plasmodia, but the plasmodia did not grow well or migrate and never sporulated. If SDM agar was used plasmodia frequently crawled out of the wells and fused with others; some plasmodia eventually crawled out of the tray altogether. Vaseline containing $ZnSO_4$ which is negatively chemotactic for P. polycephalum (personal communication from M. Williamson, this department), was smeared round the top of the wells. This prevented plasmodia migrating out of the wells, but they still did not sporulate. It appeared that plasmodia were unable to migrate and form veins in such a small area. The small area alone, however, could not account for their inability to sporulate since small sections of starving plasmodia sporulated on very small pieces of filter paper left over after the largest part of the plasmodium had been removed for radioactive experiments (Figs 15, 16 etc). Likewise plasmodia do not necessarily need to be well established growing plasmodia prior to starvation for sporulation to occur since on one occasion a plasmodium derived from an amoebal plaque on LIA, which does not support growth of plasmodia, was observed to sporulate after several days on the LIA; forming only two fruiting bodies. In theory, therefore, it seemed possible that amoebal clones could be screened on DSDM agar in multiwelled plates but in practise this was not the case. This probably was due to a combination of factors; the agar drying out, the plasmodia not properly established and the area too small for the plasmodia to migrate and form veins.

Established plasmodia obtained from mutagenised amoebae were therefore screened for their ability to sporulate in petri dishes of DSDM agar. By screening plasmodia in this way several were obtained which either did not sporulate at all, or showed a decreased ability to sporulate and in one case, mentioned previously, formed abnormal sporangia. Care had to be taken to maintain separate growing plasmodial cultures whilst plasmodia were screened for sporulation since if cultures did not sporulate on agar they died and were then lost.

ii) Genetic analysis

Plasmodia were therefore available which were unable to sporulate. These had been obtained both from mutagenesis and from cultures which had been extensively subcultured. By conducting genetic analysis of these strains it was hoped to determine whether the Spo^- phenotype was caused by a single mutation. Amoebae of the Spo^- strains (obtained as described in Methods, Section 4e,ii) would be crossed with amoebae of a strain which it was assumed sporulated normally. Amoebae of the strain LU862 were chosen for the cross since this strain carries the correct matA and rac (matB) alleles to allow crossing with the CL strain and also carries a different fus allele which facilitates selection of crossed plasmodia from plasmodia arising apogamically. The strain LU216 would also have been suitable. It was presumed that amoebae from LU862 would not carry any spo mutations ie. were potentially Spo^+ . These would be crossed with amoebae of the Spo^- strains and the resulting progeny would be analysed for sporulation capacity. Before this type of cross was attempted a cross between the strains LU216 and LU276 was carried out as a check on the procedure for carrying out crosses and analysing the progeny. Progeny from the plasmodia obtained from crossed amoebae were tested for their ability to form plasmodia in clones;

the plasmodia obtained from clones were then screened for colour, fusion genotype and whether they carried the npfA1 allele (Table 23). From table 23 or fig 25 it can be seen that the ratio of segregation of the alleles matA, whi and npfA1 were approximately 1:1 indicating they were unlinked. However, of the 21 matAh plasmodia obtained 14 were fusA2 and only 7 fusA1 suggesting that some linkage to matA might be affecting segregation of the fus allele in the progeny. Because of difficulties in determining whether plasmodia were actually growing in the presence of leucine only 15 of the 21 plasmodia obtained were successfully screened for leucine requirement and of these 11 were leu⁺ and 4 leu-1 which again suggests some linkage influencing segregation of the leu allele. Before carrying out the cross between CL (Spo⁻) and LU862 (presumed Spo⁺) it was decided to check the viability of the spores produced by the CL strains being used. CL strains produce spores which are mainly non-viable (Laffler & Dove, 1977) whilst diploid plasmodia produce much higher percentages of viable spores. Consequently two CL subcultures and the newly formed diploid plasmodium LU216 x LU276 were used to check viability of spores produced (Table 24). Clearly the CL plasmodia used, whether sporulating well or poorly, were producing mainly non-viable spores, confirming that experiments were being conducted with haploid plasmodia.

The cross between CL (Spo⁻) and LU862 (presumed Spo⁺) was then carried out. The Spo⁻ strain chosen was subculture C (Table 13) which never sporulated after illumination at 72 h. Since it had been subcultured further since conducting the experiments in Table 13, it was in fact no longer sporulating at all even after further illumination. This was also the strain used to examine DNA synthesis in an asporogenous strain (Figs 17, 18). Progeny from the cross CL (Spo⁻) x LU862 (presumed Spo⁺) segregated 1:1 for the matA allele (Table 25). However when plasmodia produced by those amoebae carrying matAh were tested for their

Table 23

Analysis of progeny of cross LU216 x LU276

Amoebae of the strains LU216 and LU276 were crossed.

The plasmodium which was formed was allowed to sporulate and the resulting amoebal progeny were analysed for their ability to form plasmodia in clones. Plasmodia obtained from clones were screened for colour, fusion genotype and whether they carried the npfA-1 or leu-1 alleles.

Table 23

Genotypes of amoebal progeny formed in clones				No. in class
<u>matA</u>	<u>npfA</u>	<u>fusA</u>	<u>whi</u>	
h	+	2	+	6
h	+	1	+	1
h	+	2	1	2
h	+	1	1	2
h	1	2	+	3
h	1	1	+	1
h	1	2	1	3
h	1	1	1	2
				21
3	ND	ND	ND	25

ND not determined

TOTAL 46

Of the above 21 amoebal progeny which carried the matAh allele, 15 were screened for leucine requirement, their genotypes were:-

<u>matA</u>	<u>npfA</u>	<u>fusA</u>	<u>whi</u>	<u>leu</u>	No. in class
h	+	2	+	+	5
h	+	2	+	1	1
h	+	2	1	+	1
h	+	1	1	1	1
h	1	2	+	+	2
h	1	2	+	1	1
h	1	1	+	1	1
h	1	2	1	+	1
h	1	1	1	+	2

Figure 25

Summary of results of cross, LU216 x LU276

Parental genotypes:

LU216 matA3, fusA1, rac-2

LU276 matAh, fusA2, rac-1, npfA1, leu-1, whi-1

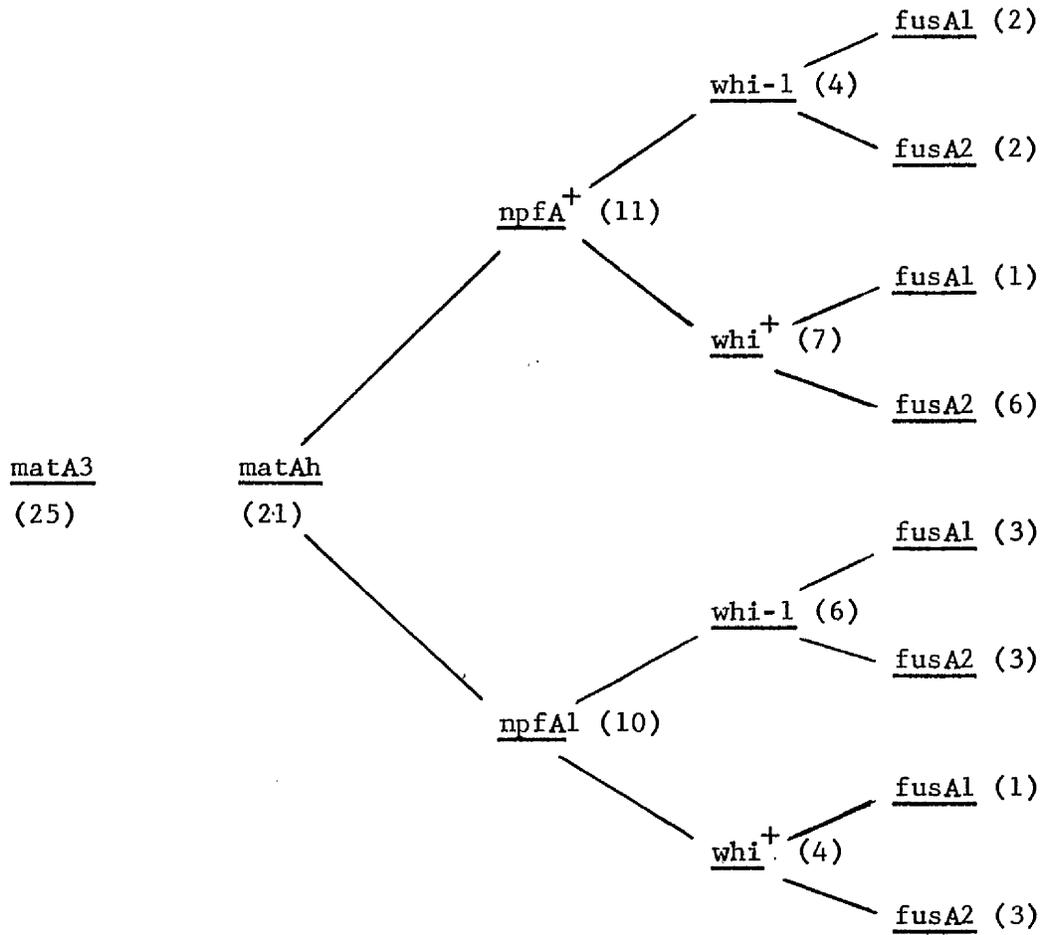


Table 24

Spore viabilities

Plasmodia were allowed to sporulate on DSDM agar. Sporangia were harvested and spore suspensions prepared (see Methods section). The number of spores present which were viable was calculated by the procedure described in the Methods section 6.

Strain	Viability of spores produced %	Sporulation frequency on DSDM agar
CL (a)	0.58	4/4
CL (b)	0.64	2/4
LU216 x LU276	78.6	4/4

(a) equivalent to subculture A in Table 13

(b) " " " D in Table 13

Table 25

Sporulation frequency of progeny from Spo⁻ x Spo⁺ cross

CL x LU862 matAh, rac-1, fusA2, Spo⁻ x
 matA3, rac-2, fusA1, Spo⁺

Amoebae from the above two strains were crossed and the resulting diploid plasmodium (distinguished from selfed plasmodia by the fusion test - see Methods section 5b) sporulated normally. Amoebal progeny from this plasmodium were screened for the matAh allele and their subsequent ability to sporulate.

genotype of progeny		No. in class
matA	Spo	
h	+	0
h	-	14
3	ND	15

ND not determined

ability to sporulate none of them were able to do so (Table 25). The diploid crossed plasmodium was, however, capable of sporulating normally which meant that if LU862 itself carried a spo allele then complementation between this and the spo allele carried in the CL amoebae must have occurred. Another possibility to account for the lack of Spo⁺ progeny would be if the spo marker carried by the CL strain was closely linked to matA and so was inherited by all the matAh progeny amoebae. Results from this cross were therefore inconclusive.

Another method of testing whether different mutations were involved in the loss of sporulation would be to test for complementation of mutations in heterokaryons formed by fusion of plasmodia. Although complementation between putative sporulation mutants obtained after mutagenesis was not tested, four subcultures of CL were chosen for their varying ability to sporulate (Table 26) and the ability of heterokaryons formed from these plasmodia to sporulate was investigated. Fusion of a poor sporulating culture with a good sporulating culture was not capable of producing a plasmodium which sporulated well (table 26). In fact, in all cases the resulting plasmodium displayed the same sporulation capacity as the poorer plasmodium prior to fusion. Fusion of plasmodia showing intermediate sporulation capacity did not produce plasmodia which sporulated well. Consequently no complementation was seen to occur (table 26) and it could be concluded that all these plasmodia had lost the ability to sporulate in the same way, possibly by the same mutation, and poor sporulators dominate good sporulators in fusion experiments (table 26).

Table 26

Subcultures

1. Newly prepared from spherules produced from subculture A in table 13, spherules 7 months old.
2. Subculture A in table 13, since subcultured and beginning to lose the ability to sporulate.
3. Newly prepared from the same spherules used to produce subculture B in table 13, spherules 21 months old.
4. Subculture C in table 13, since subcultured and no longer able to sporulate at all. This subculture was used in the DNA synthesis expts. (Figs 17, 18) and in the $Spo^- \times Spo^+$ cross (Table 25).

Table 26

Sporulation capacity of plasmodia formed by fusion of plasmodia
with differing sporulation abilities

Plasmodia were allowed to fuse on SDM agar; sporulation capacity of the resulting plasmodium was tested on DSDM agar.

<u>Plasmodium</u>	<u>Sporulation frequency</u>
1	5/5
2	4/5
3	3/5
4	0/5
1/2	4/4
1/3	3/4
1/4	0/4
2/3	3/4
2/4	0/4
3/4	1/4

DISCUSSION

1. Requirements for sporulation of *P. polycephalum* CL

The initial aim of this work was to obtain a method of reliably producing sporulation of all cultures of the CL strain of *P. polycephalum*. Plasmodia of the CL strain gave the best sporulation frequency when they were formed from microplasmodia which had been harvested at the end of exponential growth, this confirms what had previously been reported for the diploid M₃C strain (Daniel & Rusch, 1962; Daniel & Baldwin, 1964). It was noticed that this requirement could be overcome to some extent if the microplasmodia were allowed to fuse and grow as a surface plasmodium for 24 h prior to starvation; this was particularly true of very young cultures of microplasmodia (Tables 5 & 6). Presumably in this case the extra 24 h growth allowed these plasmodia to reach a state similar to that of the late exponential growth phase microplasmodia (generally harvested after 72 h growth as microplasmodia). In general sporulation frequency of microplasmodia harvested after 48 h growth was improved, by overnight growth as a surface plasmodium, to a greater extent than that of those harvested after 24 h growth. However, 24 h growth as a surface plasmodium could also improve sporulation frequency of older microplasmodial cultures (Table 6). Presumably therefore this growth as a surface plasmodium prior to starvation somehow improves the ability of the plasmodium to respond to the stimulus of starvation and so ultimately to respond to illumination and sporulate. On the whole, however, late log phase microplasmodia harvested for sporulation gave the best results not only sporulating with higher frequency but also earlier than plasmodia formed from younger or older microplasmodia. Growth as a surface plasmodium prior to starvation would be advantageous if larger amounts of material were required for study since the plasmodial mass increases considerably during the 24 h growth period. Furthermore it would be

useful if for example it was desirable to initiate starvation at particular stages of the cell cycle. Finally as already mentioned, it would allow microplasmodia to be harvested over a less restricted period of the growth cycle; otherwise harvesting microplasmodia at the end of exponential growth gives best sporulation frequencies.

It was not clear whether this ability of late log cultures to sporulate better than younger cultures was a result of the different number of microplasmodia which would be contained in the standard volume harvested at different times. Obviously those samples from early in the life cycle would contain far fewer microplasmodia. By standardising the mass of microplasmodia harvested at the various times during the growth cycle it was confirmed that their mass was not responsible for the differences in sporulation frequency. The age of the microplasmodia when harvested therefore, determines whether any plasmodium they form will sporulate if immediately exposed to starvation.

It was further confirmed that the volume of microplasmodia harvested had no effect on sporulation frequency. Varying the volume harvested at the end of exponential growth, when sporulation potential was at its peak, was shown to have no effect on whether sporulation occurred (Table 7). This has previously been shown to be true of the diploid M₃C strain (Daniel & Rusch, 1962a). The only real consequence of changing the volume of microplasmodia harvested was on the number of fruiting bodies ultimately formed, since increasing the volume harvested increased the number of fruiting bodies. The number of fruiting bodies formed is therefore, directly proportional to the mass of the plasmodium from which they have developed.

Recently it was suggested (Wilkins & Reynolds, 1979) that an important factor in determining whether a plasmodium would sporulate was the ratio of plasmodial mass to starvation medium volume. This it

was suggested was due to a factor which plasmodia produced during starvation and transported into the surrounding medium; the concentration of this factor determined when and if sporulation occurred. Using a diploid CL plasmodium formed by heat shock they demonstrated that larger plasmodia sporulated with higher frequency than smaller ones on similar volumes of medium, whilst reducing the volume of medium underlying a given size of plasmodia would increase sporulation frequency. In the work reported here similar experiments were conducted using the haploid CL strain but as all plasmodia sporulated on all occasions, varying the ratio of microplasmodia harvested to volume of medium had no effect (Table 7). Wilkins and Reynolds (1979) furthermore reported that successive transfers to fresh SM during starvation either prevented or delayed sporulation since the level of the factor was being depleted with each transfer to fresh medium. Transfer of the CL plasmodia to fresh sporulation medium during starvation had no effect either on sporulation frequency or on time of sporulation (Table 8) even when plasmodia were transferred sequentially at 24, 48 and 72 h during starvation. Consequently no evidence to support the idea that starving plasmodia produce and transport a factor into the medium was obtained from the haploid CL strain of P. polycephalum.

In most previous reports of sporulation of P. polycephalum a starvation time of 96 h in the dark prior to illumination was used, although suggestions of earlier sporulation were made (Daniel & Rusch, 1962a; Wilkins & Reynolds, 1979) but shorter incubation in the dark considerably reduced the number of cultures successfully sporulating. The length of starvation required by the haploid CL strain had not been determined. Before defining the length of starvation it was first necessary to define when starvation was being initiated. It was found that the period of coalescence or fusion of microplasmodia previously

reported necessary to form a plasmodium, generally a period of several hours in the absence of medium, was not required. Sporulation frequencies remained unchanged - all sporulated - when sporulation medium was introduced beneath the microplasmodia immediately after they had been spread onto the filter. Starvation medium therefore was added immediately after microplasmodia had been transferred to filter papers.

After this it was shown that 72 h starvation in the dark was required before all plasmodia could be induced to sporulate by one light pulse (Table 9). Shorter periods of starvation prior to illumination never resulted in 100% sporulation. It was however noticed that after about 60 h starvation an increasing number of plasmodia became capable of sporulation. Daniel and Rusch (1962a) reported that although the M_3C strain required 4 days starvation before illumination, a few cultures would sporulate if illuminated after only 3 days starvation. It would therefore appear that starving plasmodia do not follow a strictly defined timetable since some plasmodia invariably sporulate earlier than others. In the case of the CL strain, illumination between 60 and 72 h would produce sporulation of some cultures whilst others would not be capable of responding to illumination. It would seem that a certain event, or events, must take place during the starvation period before the starved plasmodium becomes capable of responding to light. After this event or events have occurred, a plasmodium will sporulate as soon as it is exposed to light. There is however a time limit on how long the plasmodium in this state can wait for light, and if the required light pulse does not occur then plasmodia die and are no longer capable of sporulation (Table 9). In particular plasmodia left for 120 h or longer failed to sporulate. For the M_3C strain, which was illuminated at four days, sporulation competence was maintained until the 7th day of starvation (Daniel & Rusch, 1962a).

It is suggested above that an event which occurs between 60 and 72 h during starvation allows the plasmodium to respond to light. Sometimes, however, this event appears to occur earlier, since starved plasmodia given light pulses at 24 h intervals throughout starvation were observed in some cases to sporulate after only 36 and 42 h starvation (Fig 6). In all experiments conducted in this manner, some plasmodia sporulated earlier than others and in some experiments all plasmodia had sporulated before being given the light pulse at 72 h (Fig 6b,c). Two main points can be derived from the information given in Fig 7; first if any group of plasmodia are given several light pulses some will invariably sporulate earlier than others. This again stresses the lack of any strictly defined timetable of events during starvation, although a certain amount of the asynchrony may be caused by plasmodia being derived from different cultures of microplasmodia (see later in discussion). In all cases, however, a light pulse given at 72 h will induce sporulation of any remaining unsporulated cultures. Second, several light pulses given during starvation seemed to be more effective in producing earlier sporulation than one light pulse. In Fig 6b complete sporulation of all 10 cultures in the experiment was achieved by 60 h whilst of the 4 cultures left 60 h in the dark before one light pulse, only one of the four sporulated after illumination (Table 9). Periodic illumination would only really be advantageous if the only desired result was for all cultures to sporulate. When cultures are being compared and attempts to define events during starvation are being made, periodic illumination of a series of cultures is inadequate since it increases the degree of asynchrony of events between starving plasmodia. It was estimated that under these conditions approximately 50% of the plasmodia involved will always sporulate earlier than others. By leaving

all cultures for 72 h in the dark and then giving one light pulse, all cultures sporulated within 24 h of illumination. This allows all cultures to reach a state where they are capable of responding to illumination. Events after illumination are therefore controlled, as all plasmodia behave identically after illumination and sporulate at the same time.

Illumination was an absolute requirement, since cultures left continuously in the dark never sporulated (Table 10). Once again, of cultures illuminated periodically or left continuously in daylight almost 50% sporulated before illumination at 72 h. After 72 h incubation in the dark plasmodia only required a very short light pulse to induce sporulation (Table 11), after 5 min illumination all cultures sporulated. It would therefore appear that although illumination is required, only a very brief exposure to light is necessary if cultures have been starving for 72 h in the dark beforehand.

An attempt was made to confirm that light of wavelengths in the region 350-500 nm would induce sporulation (Daniel & Rusch, 1962a; Daniel & Baldwin, 1964). Only light of wavelength 400 nm was observed to induce any sporulation (Table 12) when starved cultures were exposed to light of this wavelength after 72 h incubation in the dark. However, this light never produced the 100% sporulation obtained with control white light. This may have been due to the fact that the intensity of light produced by the monochromator was much lower than that of the white light. This would suggest that the intensity of the light was insufficient to produce sporulation. Intensity of light used for illumination was found to affect sporulation of the M₃C strain, in that lowering the intensity reduced sporulation frequency (Daniel & Rusch, 1962a). The light of wavelength 400 nm was, however, able to produce

some sporulation even although this wavelength gave the lowest intensity reading, suggesting at least that wavelengths around 400 nm are most likely to be required to produce sporulation. The variation in intensity of the light did, however, limit the conclusions which could be drawn from these experiments.

2. Loss of ability to sporulate

Although using the above system, plasmodia of P. polycephalum CL could reproducibly be induced to sporulate, one other factor had to be considered. During the course of deriving the system used to produce sporulation it became apparent that differences existed between the various subcultures of the one strain which were available. In some subcultures sporulation occurred later ie. after further illumination at 96 h but not after illumination at 72 h, whilst others failed to sporulate even after a second period of illumination. When several subcultures were compared for their ability to sporulate, considerable differences were observed (Table 13). Poor sporulation appeared to be arising after extensive subculture as microplasmodia, such that not all of the CL subcultures available were able to sporulate after illumination at 72 h. This loss of ability to sporulate has been reported previously (Daniel & Baldwin, 1964; Hosoda, 1980). Consequently before this system to produce sporulation could be used in another laboratory it would first have to be established that any culture being used was sporulating well and had not lost the ability to sporulate as a result of extensive subculture. In the course of this work single subcultures were observed to change from good sporulators to cultures which sporulated late and ultimately to cultures which did not sporulate at all.

Clearly this will affect interpretation of results since if a culture does not sporulate reliably, certain conditions may improve sporulation frequency whilst these conditions will have no effect on a culture which is already sporulating on all occasions. This is true of results produced by Wilkins and Reynolds (1979) since these authors very rarely obtained 100% sporulation and had therefore to rely on overall increases or decreases in sporulation frequency to assess the importance of various factors. The main suggestion from these authors was that plasmodia produced a promoting factor, the level of which is ultimately responsible for producing sporulation. This was deduced from experiments which indicated that conditions which favoured production of, or concentration of the factor caused cultures to 'sporulate with higher frequency', they still, however, did not report 100% sporulation. This theory is difficult to check with a culture which sporulates all the time regardless of these changes in condition (Tables 7, 8). Wilkins & Reynolds (1979) suggested that the requirement for niacin during starvation was stimulatory rather than strictly essential and demonstrated that diploid CL plasmodia could sporulate, albeit in a delayed fashion, in the absence of niacin. Two possible explanations were considered: either the factor produced by the plasmodium and niacin were stimulating sporulation by different pathways or niacin in some way boosted the production of this factor (Wilkins & Reynolds, 1979). Niacin, therefore, would not be required if sufficient factor was already being produced. In the present work subcultures of the haploid CL strain which sporulated on all occasions were found not to require niacin but this was not true of the subcultures which sporulate with poorer frequencies (Table 13). Furthermore it was noticed that increasing the concentration of niacin could improve the sporulation frequency of poor sporulating subcultures in much the same way as medium containing the factor was reported to

improve sporulation frequencies of the diploid CL plasmodia (Wilkins & Reynolds, 1979). It seems possible therefore that either by introducing the factor directly into the medium or by increasing niacin concentration, sporulation frequencies can be improved. If the culture chosen, however, sporulates on all occasions then neither course of action can improve on the 100% sporulation readily obtained. This suggests that these cultures may produce extremely high levels of this factor which do not require additional niacin for their production, while sporulation frequencies of poorer subcultures may be enhanced either directly by adding this factor or by adding niacin.

Consequently it would seem important that the sporulation status of a culture is clearly defined before presenting results relating to sporulation of P. polycephalum since considerable variations in requirements may arise as the subculture loses its ability to sporulate. All of the experiments presented in the present work were conducted, unless otherwise stated, on cultures which gave 100% sporulation after illumination at 72 h during starvation.

3. Time of commitment to sporulation

Plasmodia did not become committed to sporulation until between 4 and 5 h after illumination, after which time they sporulated regardless of nutrition (Table 15). At any time prior to this they could resume growth if transferred back onto growth medium. Earlier workers had first established that plasmodia became committed to sporulation after illumination (Daniel & Rusch, 1962a) and later it was deduced that the time of commitment, of the M₃C strain, was about 3 h after illumination (Sauer et al, 1969a). In the work reported here it was further demonstrated that the time of commitment was directly related to the time at which the starved plasmodia were illuminated,

since when the time of illumination was changed a similar change in the time of commitment was observed (Fig 7). Illumination would therefore seem to trigger events leading to the commitment of the plasmodium to sporulation. Even after illumination, plasmodia can still be prevented from sporulating if re-introduced to nutrient, but if the series of events triggered by light are allowed to proceed uninterrupted then the final event committing plasmodia to sporulation occurs 4 to 5 h after illumination.

Further investigation suggested that a carbon source is required if sporulation is to be prevented since D-glucose and L-glutamate were as effective as complete growth medium in preventing sporulation. A nitrogen source alone was incapable of preventing sporulation, $(\text{NH}_4)_2\text{SO}_4$ failed to prevent sporulation whilst, although L-glutamine could not prevent sporulation, it could delay it. Carbon starvation would therefore seem to be required for sporulation of P. polycephalum, and in this respect it differs from the yeast S. cerevisiae. In S. cerevisiae sporulation is induced by transfer to a nitrogen free medium, NH_4^+ ions have been shown to be capable of preventing sporulation if added either in the form of $(\text{NH}_4)_2\text{SO}_4$ or glutamine (Pinon, 1977). It is suggested that NH_4^+ ions may interfere with the unfolding of the genetic program of sporulation such that, depending on when NH_4^+ ions are added, the next step in the sequence would be prevented (Pinon, 1977). A similar sequence of events may occur in sporulation of P. polycephalum but the interference of progression through this sequence is brought about by a carbon and not a nitrogen source.

The above results certainly indicate a two stage process whereby events during the initial starvation period render the plasmodium competent to respond to illumination, which then triggers a second

series of events leading to commitment of the plasmodium to sporulation (Table 27). In the first stage therefore the carbon source must prevent sporulation by interfering with the steps which lead to the plasmodium becoming capable of responding to light, whilst after illumination it must prevent the steps which lead to the final commitment of the plasmodium to sporulation. It may be that in both cases a similar mechanism is employed.

4. Mitosis and DNA replication during starvation

As discussed in the Introduction, it was hoped to investigate the importance of DNA replication, mitosis and general progression through the cell cycle during differentiation. By using inhibitors of DNA replication and mitosis it was hoped to demonstrate whether sporulation of P. polycephalum CL was dependent on these events occurring ie. if these events were inhibited would sporulation be prevented?

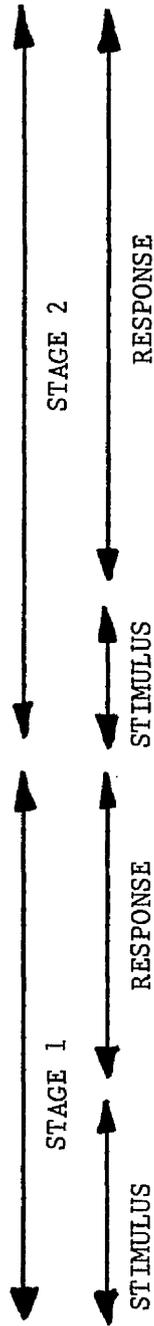
The mitotic inhibitor colchicine had previously been reported to delay mitosis in P. polycephalum (Hagino et al, 1978) but in the present study colchicine was found to have very little effect on growth of microplasmodia, even when relatively high concentrations were used (Fig 8). This is in agreement with more recent evidence which suggests that microtubule structure and function in Physarum, as in other lower eukaryotes, are more susceptible to the action of nocodazole than colchicine (Quinlan et al, 1981). Nocodazole effectively prevented growth of microplasmodia at a concentration of $20 \mu\text{gml}^{-1}$ (Fig 9) and also prevented sporulation if added at any time prior to 48 h during starvation (Table 18). After 48 h, plasmodia began to escape the effect of the drug, which would suggest that the last event during starvation that is susceptible to nocodazole, and necessary for sporulation, occurs between 48 and 72 h during starvation. This event could

Table 27

Events during starvation.

As a result of experiments designed to determine length of starvation required prior to illumination and time of commitment to sporulation, the times and events noted below appear to be significant in sporulation of P. polycephalum CL.

Time (h) during starvation	0-60	60-72	72	76-78	78-96
Events during starvation	starvation	plasmodia becomes responsive to light	light	plasmodia becomes committed	sporulation completed, morphogenesis



be a final mitosis necessary for sporulation or, as discussed in the introduction, there is a possibility that some other event involving cytoplasmic microtubules occurs at this time and this is being blocked by the presence of nocodazole. An event sensitive to nocodazole does however occur at about this time during starvation. There did not, however, appear to be a set time between 48 and 72 h when all plasmodia had completed this nocodazole sensitive event; rather there seemed to be considerable asynchrony between plasmodia, some having completed this event by 48 h whilst others were still affected by the inhibitor at 65 h (Table 18). All were unaffected by the presence of nocodazole added immediately prior to illumination at 72 h. This nocodazole sensitive event would therefore seem to occur between 48 and 60 h during starvation. Some plasmodia, therefore, complete this event considerably earlier than others and this may be related to the ability of some plasmodia to respond to illumination earlier than others. Consequently, this event may be necessary for the plasmodium to become capable of responding to light and those plasmodia which complete the event early may as a consequence be able to respond to illumination earlier than 72 h. All cultures, however, regardless of when this event takes place are able to respond to illumination at 72 h. It would have been advantageous to have confirmed that this event was in fact a mitosis by direct microscopic examination. However, as explained in the Results, this was not possible. Furthermore supposing that a mitosis did take place between 48 and 60 h during starvation it was not apparent from these results whether this was the only mitosis during starvation, since any mitoses occurring earlier would be masked by the inhibition of the final mitosis during starvation. It was also noticed that in the culture which was beginning to lose the ability to sporulate and was exhibiting delayed sporulation different results from the nocodazole experiments were obtained. These cultures

sporulated after illumination at 96 h but not at 72 h. Addition of nocodazole at 72 h prevented sporulation. This suggests that this last nocodazole sensitive event does play a role in preparing the plasmodium to respond to light, such that when it was delayed, occurring after 72 h starvation instead of 48 h, plasmodia were unable to respond to illumination until 96 h instead of at 72 h as normal.

Similar experiments were conducted using inhibitors of DNA replication. The first of these inhibitors tested, AraC, like the mitotic inhibitor colchicine had no effect on growth of microplasmodia (Fig 10). Hydroxyurea when tested as an alternative proved to be an efficient inhibitor of growth of microplasmodia (Fig 10). Hydroxyurea also prevented sporulation if added any time prior to 24 h during starvation (Table 20); plasmodia escaped the effect of the drug between 24 and 48 h during starvation. This suggests that the last DNA replication occurs anything up to 24 h before the last mitosis, if it is assumed that the final nocodazole sensitive event is a mitosis. From this assumption it can next be suggested that the last mitosis during starvation is not immediately followed by DNA replication. When exactly the last DNA replication occurred again seemed to vary between plasmodia, since some plasmodia escaped the effect of hydroxyurea before 24 h in starvation whilst the majority appeared to escape the effect of the drug between 24 and 48 h in starvation (Table 20). It did however seem likely that the last DNA replication during starvation occurred between 24 and 48 h and the time at which it occurred determined in turn when the last mitosis (or nocodazole sensitive event) occurred between 48 and 60 h.

The effect of BUdR on sporulation was also tested. Low concentrations of this inhibitor, which had very little effect on growth, were able to prevent sporulation if added any time prior to 48 h during

starvation. This is in agreement with other reports of BUdR having a greater inhibitory effect on differentiation than on growth (Rutter et al., 1973). The timing of the effect of BUdR differs from that of hydroxyurea suggesting that the effect of the former may not be directly on DNA replication, which is compatible with the fact that these low concentrations of BUdR have no effect on growth of microplasmodia. Furthermore, as discussed below, low concentrations of BUdR have no effect on uptake of [methyl-³H] thymidine into growing plasmodia, again suggesting that DNA replication is not affected. These concentrations are, however, capable of preventing sporulation (Table 20) and further work on the effect of BUdR on sporulation might yield more information on the mechanism of action of this inhibitor as well as more information on the process of sporulation. The timing of the effect of BUdR on sporulation in fact resembles that of the microtubule inhibitor nocodazole more than it does that of hydroxyurea (Tables 18 & 20). If as discussed in the introduction the main effect of BUdR is on transcription, then this escape from BUdR inhibition at about 48 h during starvation may reflect some change in the pattern of transcription associated with the last nocodazole sensitive event during starvation.

Results from experiments involving escape from hydroxyurea inhibition of sporulation suggested that the last DNA replication during starvation occurred between 24 and 48 h. It was decided to try and place the time at which this replication occurred more accurately by measuring uptake of [methyl-³H] thymidine into acid-insoluble material during starvation. The method used was first checked by measuring uptake into growing plasmodia, a peak of uptake of thymidine, which was assumed to be associated with DNA replication, was readily demonstrated (Fig 12). Uptake lasted 2 to 3 h, peaks being separated by a gap of

6 to 8 h. This agrees with previous data suggesting an overall cell cycle of 8 to 10 h including an S phase of 2 to 3 h (Nygaard et al, 1960; Braun et al, 1965). The presence of excess uridine in the radioactive medium had no effect on incorporation (Fig 13), which demonstrated that uptake was probably not affected by incorporation of $[^3\text{H}]$ label into RNA as well as DNA. Uptake of $[^3\text{H}]$ thymidine into growing plasmodia was used, as an alternative method to effect on growth of microplasmodia, to test the effect of the inhibitors used in this work. The DNA inhibitor used, hydroxyurea, completely prevented incorporation of labelled thymidine, but the mitotic inhibitor, nocodazole, had no effect. High concentrations of BUdR appeared to completely prevent DNA replication, but low concentrations although not completely preventing incorporation, did cause a reduction in the amount of $[^3\text{H}]$ incorporated. This presumably was due to BUdR competing with thymidine for incorporation into DNA; consequently it is difficult to determine whether the high concentration of BUdR prevents DNA replication or just prevents uptake and incorporation of the radioactive label by direct competition. Further experiments, possibly using an alternative label to $[^3\text{H}]$ thymidine, might clarify whether high concentrations of BUdR do inhibit DNA replication, or merely compete with $[^3\text{H}]$ thymidine for incorporation.

In four separate experiments a peak of incorporation of $[^3\text{H}]$ thymidine was observed between 20 and 24 h (Fig 16). It was noticeable, as predicted earlier, that the time of this replication was not exactly synchronous between different plasmodia (Fig 16). However of the four plasmodia tested two were formed from the same culture of microplasmodia (Fig 16b,c) and good synchrony was observed between these two plasmodia, replication occurring between about 23 and 25 h. Consequently it would appear that plasmodia formed from the same culture of microplasmodia

retain synchrony of cell cycle events until at least 24 h into starvation. Plasmodia formed from different cultures of microplasmodia, although all replicating their DNA at about 24 h into starvation, do show differences of several hours in the time at which this replication starts. This may in some way help to explain why some plasmodia are capable of responding to light earlier than others ie. some plasmodia may complete a crucial cell cycle event earlier than others and so become capable of responding to light. After the replication at about 24 h, no further replication was observed up until 50 h in starvation (ie. no further replication was observed in the 24 h after plasmodia began to escape inhibition of sporulation by hydroxyurea). Plasmodia were not tested for incorporation of $[^3\text{H}]$ thymidine after 50 h mainly for practical reasons. It seemed unlikely, however, that any replication occurred after this time since by 50 h the majority of plasmodia were no longer affected by hydroxyurea. Plasmodia were however checked for $[^3\text{H}]$ thymidine incorporation between 65 and 72 h into starvation, ie. immediately before illumination. Sauer et al (1969a) had suggested that mitosis occurred at about 4 h before illumination and was followed by DNA replication in the M_3C strain which was illuminated at 96 h. No uptake of $[^3\text{H}]$ thymidine was observed in the period immediately before illumination, it was therefore assumed that the last DNA replication during starvation occurred at about 24 h, some 48 h prior to illumination. This it was assumed was followed by a final mitosis between 48 and 60 h in starvation (assuming the last nocodazole sensitive event is a mitosis).

Although DNA replication had been shown to occur at about 24 h during starvation it was not known whether this was the last or the only replication during starvation. Further investigation revealed that this was the third replication to take place during starvation. Earlier in

starvation replication occurred at about 4 h and 15 h before the third replication at about 24 h. After fusion of microplasmodia to form a plasmodium, the first mitosis followed by DNA replication would be expected at about 4-5 h, this being the arithmetic average of all the cell cycle times likely to be represented in the newly formed plasmodium (Guttes & Guttes, 1964). It would seem therefore that during the first 24 h of starvation the plasmodium replicates its DNA and undergoes mitosis at the normal times in a cell cycle of 10 to 12 h. After the third replication the plasmodium enters an extended G₂, events during this G₂ period seem to be dependent on the last replication having been completed and ultimately lead to the plasmodium being able to respond to illumination.

Results from the experiments with nocodazole had suggested that the time of the last nocodazole sensitive event was directly associated with the time that the plasmodium became capable of responding to illumination, since a plasmodium showing delayed sporulation also showed a delay in the time at which it escaped from nocodazole inhibition of sporulation. When $[^3\text{H}]$ thymidine incorporation was compared in a normal sporulating culture and one which could not sporulate the first two DNA replications occurred normally. The third peak of incorporation of $[^3\text{H}]$ thymidine was however only observed in the sporulating culture. In the asporogenous culture this replication was either absent or delayed beyond 32 h in starvation. This once again suggests that cell cycle events do play a role in determining which cultures are able to sporulate, such that unless the third replication occurs plasmodia cannot enter the extended G₂ from which competence develops.

Thus DNA replication and mitosis are apparently required for sporulation, or at least for plasmodia to become capable of responding to illumination. However, whether they are actually required for

development depends on when development and morphogenesis are considered to begin. Plasmodia are not committed to sporulation until 4 to 5 h after illumination whilst no signs of morphogenesis are observed until some hours later again. Consequently the last DNA replication occurs some 52 h and, assuming the last nocodazole sensitive event is a mitosis, the last mitosis 26 h before the plasmodium is committed to sporulation. These cell cycle events appear to play a crucial role in producing a plasmodium which is capable of responding to illumination.

For the next part of the discussion it will be assumed that the last nocodazole sensitive event in starvation is a mitosis. This seems a reasonable assumption, since the spindle microtubules are the main target for nocodazole at all other times, and there is no other evidence to suggest that an event in which cytoplasmic microtubules play a part occurs at this time. No change in appearance or movement of the plasmodium takes place at this time; hence it will be assumed that after an extended G2 the plasmodium enters a final mitosis which is not followed by DNA replication. Consideration, therefore, of events during starvation prior to illumination reveals that the first two cell cycles proceed normally, however after the third mitosis and DNA replication changes begin to be triggered as the plasmodium enters an extended G2 period in excess of 24 h long, more than twice the normal length of G2. After this G2 the plasmodium enters a final mitosis which is different from all others in that it is not immediately followed by DNA replication. The last detectable DNA replication and the putative last mitosis in starvation are therefore important, this is stressed by the variation seen in these events in plasmodia unable to sporulate. Table 28 details cell cycle events in the first stage of sporulation (as described in Table 27). The extended G2 prior to the last mitosis

Table 28

Cell cycle events during the first stage of sporulation

Presumed cell cycle events as deduced from experiments with hydroxyurea and nocodazole and from

[³H] thymidine incorporation.

Time (h) during starvation	0	10	20	30	40	50	60	70
Stage of cell cycle	<p>G₂-M-S — G₂ — M-S-G₂ — M-S — G₂ — M^(?) — G₀^(?)</p> <p style="text-align: right;">↑ plasmodia able to respond to light</p>							

would appear to be the result of starvation conditions which have by then existed for more than 24 h. This perhaps leads to the mitosis which is not immediately followed by DNA replication. It would follow that if the plasmodium does not enter a DNA replication then it would be in a G1 phase.

Consideration of other examples of eukaryotic differentiation has led to the belief that there is a point in G1 from which cells can either differentiate or enter another mitotic cell cycle depending on environmental conditions. Early work with temperature sensitive mutants in the yeast S. cerevisiae led to the discovery of a point called 'start' in early G1. Depending on the nutritional status of the cells, they either continued cell division or entered a pathway of differentiation from this point (Hartwell, 1974; Nurse, 1981). A similar point in G1 was described for Sch. pombe (Nurse & Bisset, 1981). More recently isolation of cell cycle ts mutants of Friend erythroleukaemia cells has been reported (Conkie et al, 1981). Using these mutants it has been demonstrated that they have a requirement for a cell cycle event before they can become differentiated and capable of synthesising haemoglobin. This cell cycle event has been shown to occur in the G1 phase of the cell cycle. Hence a similar 'start' mechanism has been described in a higher eukaryotic system (Conkie et al, 1981). This demonstrates how investigation of differentiation in lower eukaryotes can yield information relevant to general eukaryotic differentiation, and also how, by virtue of their simplicity, points may become apparent in lower eukaryotes long before similar information is obtained from their higher eukaryotic counterparts.

In considering P. polycephalum, therefore, it is possible that after the last mitosis in starvation plasmodia enter a G1, or G0

equivalent, from which they can choose to differentiate or continue mitotic cell division. The first stage of sporulation is therefore very dependent on cell cycle events. The plasmodium continues through the cell cycle until, presumably because of lack of nutrient, the plasmodium is arrested at 'start' or an equivalent of start. This point would, therefore, be the only cell cycle state from which the plasmodium could enter the second stage of sporulation, triggered by illumination. Possibly 'start' in P. polycephalum coincides with the end of mitosis rather than a point in early G1 since no G1 normally exists. Presumably from this point the plasmodium either sporulates in response to illumination or re-enters the mitotic cell cycle, by replicating its DNA, if nutrient becomes available. This alternative is open until the plasmodium finally becomes committed to sporulation.

Much of the above was deduced from results obtained by use of inhibitors of mitosis and DNA replication. As mentioned previously when using nocodazole, which is a microtubule assembly inhibitor, it must not be immediately assumed that mitosis is the only process being affected, other events involving microtubules may take place. Furthermore, in the present work some attempt was made to demonstrate that microplasmodia may become resistant to the drugs or may be capable of breaking down the drugs - although the drugs may have been unstable at the temperatures at which they were incubated and not directly affected by the microplasmodia. Another consideration would be the possibility that the times at which the plasmodia became resistant to the drugs were related to changes in permeability to the drugs. Changes in permeability of plasmodia have been reported to occur after illumination (Sauer et al, 1969a), but not during the starvation period before illumination.

5. Genetic analysis of sporulation

The initial aim of the genetic part of this work was to attempt to isolate mutants which affected sporulation. Ideally mutants *ts* for cell cycle events, as have been previously isolated (Wheals et al, 1976; Gingold et al, 1976; Sudberry et al, 1976; Del Castillo et al, 1978; Laffler et al, 1979; Burland & Dee, 1980), could have been used to confirm any requirement for cell cycle events in sporulation. Temperature sensitive mutants were isolated, but none of these *ts* amoebae were found to be *ts* in the plasmodial stage, this problem has been encountered by other workers (Wheals et al, 1976; Gingold et al, 1976; Sudberry et al, 1976; Del Castillo et al, 1978; Laffler et al, 1979) and may be overcome by using the medium described by Burland and Dee (1979) on which both amoebae and plasmodia grow reasonably well. No *ts* mutants in plasmodia, therefore, were isolated, but some of the *ts* amoebae were observed to give rise to plasmodia which had defects with respect to sporulation (Table 22). Since these mutants had more than one phenotypical change it is possible that they arose as a result of several mutations, and not a single mutation. It was however hoped that by analysing crosses between these Spo^- mutants and a Spo^+ strain it would be possible to determine whether any of them were caused by a single mutation, and if so whether they were linked to any of the markers which have already been described in *P. polycephalum*.

Initially a subculture of CL which could not sporulate was crossed with the presumed Spo^+ strain (LU862). Later, it was hoped, the strains which had become asporogenous after mutation could be crossed with the Spo^+ strain. Basically, with the initial cross, the resultant, presumed Spo^+/Spo^- diploid, sporulated normally but none of the progeny were able to do so. That is, none of the tested matAh progeny could

sporulate. It was thought that either the spo marker was linked with the matAh locus or possibly the presumed Spo^+ strain was in fact Spo^- . In either case the diploid would be capable of sporulation for in the latter case this would be possible if the LU862 strain carried a different spo mutation. In the diploid plasmodium these mutations would complement one another but all of the haploid progeny would be Spo^- . Consequently a major difficulty in carrying out crosses of this kind is finding a strain with which CL can be crossed and which also has a known sporulation capacity.

Interpretation of these results was further complicated by the fact that heterokaryons formed by fusion of subcultures of the strain with varying sporulation capacity always resulted in a plasmodium which had the sporulation capacity of the poorer strain before fusion. This seemed to suggest that asporogeny was dominant. It is possible however that after fusion the number of nuclei in the cytoplasm capable of sporulation was only 50%. It may be that all the nuclei in a given amount cytoplasm must be capable of coding for a product essential for sporulation, possibly the factor suggested by Wilkins and Reynolds (1979). This would explain the apparent contradiction in that the diploid Spo^+/Spo^- or Spo^-/Spo^- plasmodia could sporulate but a haploid plasmodium with 50% Spo^+ nuclei could not, since in the case of the diploid all nuclei are effectively Spo^+ due to complementation. Consequently interpretation of the genetic analysis of sporulation was hampered by lack of knowledge of the sporulation process as a whole and inability to determine the sporulation capacity of the LU862 amoebae.

On the whole most of the original objectives of this work were satisfied. A reliable method of producing sporulation has been described and the importance of loss of sporulation capacity due to

subculture highlighted. In answering the question about the role of DNA replication and mitosis in sporulation, it has become apparent that sporulation can be divided into two distinct stages. Mitosis and DNA replication appear to play an important role in the first stage which leads to a plasmodium capable of responding to illumination. Genetically, some progress was made but more work will be required before sporulation can be completely understood genetically. It was hoped that cell cycle ts mutants could be used to clarify the role of mitosis and DNA replication in the initial stages of sporulation. Before this is possible the problem of wild type plasmodia not sporulating at the high temperature will have to be overcome. Mutants in the second stage of sporulation would also be useful to help determine what are the important events with regard to commitment and morphogenesis. This could be combined with work to clarify whether plasmodia enter a G1 phase in starvation or are arrested at a point equivalent to 'start'. For example the pattern of DNA replication after returning plasmodia, at this stage of the sporulation process, to growth medium could be studied. Would these plasmodia replicate their DNA immediately? That is are they arrested at a point at the end of mitosis from which they immediately enter S phase, on return to favourable conditions. Or is there a delay before entering S phase suggesting that ^aG1 phase might exist under these circumstances, or possibly that some other event must take place before plasmodia can return to a mitotic cell cycle. Whether 'start' has a role in the normal cell cycle of P. polycephalum could also be investigated.

It seems possible therefore that P. polycephalum GL may provide a simple and useful model for investigating a eukaryotic differentiating system both biochemically and genetically.

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APPENDIX

Appendix 1

Media

Semi-defined growth medium (SDM)

Tryptone (Oxoid)	20 g
Yeast extract (Difco)	3 g
D-glucose	20 g
KH ₂ PO ₄	4 g
N+C salts (see below)	240 ml

Each of the above was dissolved and finally made up to 2 litres in sterile distilled water. The pH was adjusted to 4.6 with 40% (w/v) KOH and the medium autoclaved at 15 lb(in²)⁻¹ for 15 min. Hemin solution (see below) was added immediately before use, 10 ml per litre of medium.

N+C salts

Citric acid	67.3 g
FeCl ₂ :4H ₂ O	1 g
MgSO ₄ :7H ₂ O	10 g
CaCl ₂ :2H ₂ O	10 g
MnCl ₂ :4H ₂ O	1.4 g
ZnSO ₄ :7H ₂ O	0.56g

Each of the above was dissolved and made up to 2 litres in sterile distilled water and autoclaved 15 lb(in²)⁻¹ for 15 min.

Hemin Solution

Hemin (Sigma) equine type III crystalline was prepared by adding 1 g NaOH to 100 ml distilled water and then adding 0.1 g hemin and autoclaving the solution at 15 lb(in²)⁻¹ for 15 min.

Semi-defined growth medium agar (SDM agar)

Equal quantities of liquid SDM (+ hemin) and molten 2% purified agar (Oxoid) were mixed.

Dilute semi-defined growth medium agar (DSDM agar)

50 ml liquid SDM (+ hemin) were added to 1 litre of molten 2% purified agar (Oxoid).

Liver Infusion Broth (LIB)

1 g Oxoid Liver Infusion powder was added to 1 litre distilled water and autoclaved $15 \text{ lb}(\text{in}^2)^{-1}$, 15 min.

Liver Infusion Agar (LIA)

1 g Oxoid Liver Infusion powder was added to 1 litre of molten 2% purified agar.

Sporulation Medium (SM)

CuCl ₂	0.048 g
KH ₂ PO ₄	0.8 g

Sporulation salts (see below) 240 ml

Each of the above was dissolved and made up to 2 litres in sterile distilled water and autoclaved $15 \text{ lb}(\text{in}^2)^{-1}$, 15 min.

Sporulation Salts (SpS)

A. Citric acid: H ₂ O	4 g
CaCl ₂ :2H ₂ O	5 g
MgSO ₄ :7H ₂ O	5 g

Each of these was dissolved in 600 ml sterile distilled water to which 0.5 ml conc. HCl had been added.

B. FeCl ₂ :4H ₂ O	0.5 g
MnCl ₂ :4H ₂ O	0.7 g
ZnSO ₄ :7H ₂ O	0.28 g

Each of these was dissolved in 50 ml sterile distilled water. A and B were then mixed and made up to 1 litre with sterile distilled water and autoclaved $15 \text{ lb}(\text{in}^2)^{-1}$, 15 min.

Immediately before use 1 ml sterile 10% (w/v) CaCO_3 and 1 ml sterile 1% nicotinamide (niacinamide, Sigma) were added per 100 ml of medium. The nicotinamide was prepared, as a 1% (w/v) solution, freshly each time medium was used.

Defined medium-1 (DM-1) (Plaut & Turnock, 1975)

	g litre ⁻¹
L-glutamic acid	3
Glycine	0.5
L-methionine	0.1
Biotin	0.005
Thiamine	0.04
Citric acid H ₂ O	3.54
Disodium EDTA	0.224
KH ₂ PO ₄	2
CaCl ₂ ·2H ₂ O	0.9
MgSO ₄ ·7H ₂ O	0.6
FeCl ₂ ·4H ₂ O	0.006
ZnSO ₄	0.034

The pH was adjusted to 4.6 with 10% (w/v) NaOH and autoclaved $15 \text{ lb}(\text{in}^2)^{-1}$, 15 min.

Immediately before use 2 ml of 20% (w/v) sterile D-glucose solution and 1 ml hemin solution were added per 100 ml of medium.

DM-1 agar

To prepare DM-1 agar equal quantities of liquid DM-1 (+ hemin and glucose) and 2.5% agar were mixed.